

THE UNIVERSITY OF LIVERPOOL

The role and regulation of AKT in CD40-mediated survival and proliferation of CLL cells

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Chapman, Elinor

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Abstract

Chronic lymphocytic leukaemia (CLL) is a proliferative disease in which malignant B lymphocytes gradually accumulate in the peripheral blood, lymph nodes and bone marrow. It is the most common leukaemia in adults and currently incurable. Novel therapeutic strategies are thus required. The serine/threonine kinase AKT has been shown to contribute to the survival and expansion of CLL cells. However, the exact role of AKT in CD40 stimulation-induced survival and proliferation of CLL cells is not well understood. The aim of this study was to investigate how AKT mediates CLL-cell survival and proliferation, in particular under the conditions that mimic the protective tissue microenvironment *in vivo*. I therefore used a co-culture system in which primary CLL cells were cultured with transfected mouse fibroblasts expressing human CD154 to model the lymph node environment where CLL cells interact with T cells through the CD40-CD154 signalling pathway, resulting in their enhanced survival and proliferation. I showed that AKT was activated in CD40-stimulated CLL cells. AKT mediated a protective effect of CD40 stimulation against cytotoxic drug (bendamustine)-induced cell death, as inhibiting AKT activity by a selective, small molecule inhibitor potentiated drug-induced killing in the stimulated cells. I also showed that AKT was required for CD40 stimulation-induced cell growth (increase in size) in CLL cells. In addition, I uncovered a selective requirement of AKT for proliferation induced by CD40 + IL-4 in CLL cells, since pharmacological inhibition of AKT significantly inhibited such proliferation in the leukaemic, but not normal B cells. In contrast, the requirement of AKT in proliferation induced by CD40 + IL-21 in CLL cells appears to be case-dependent. Further studies are thus required to understand why inhibition of AKT activity reduces CD40 + IL-21-induced proliferation in CLL cells from some but not other cases. Finally, I examined the regulation of AKT activation by the class I PI3K p110 isoforms and showed for the first time that expression of both PI3K p110 δ and p110 γ was upregulated in CD40-stimulated CLL cells. This novel observation has provided a rational basis for a future study on the function of the upregulated isoforms, in particular PI3K p110 γ isoform in stimulated CLL cells in order to establish its role in the pathogenesis of CLL. In conclusion, I have demonstrated that the PI3K-AKT pathway plays an important role in CLL-cell survival, growth and proliferation in response to CD40 stimulation, a stimulus that is highly relevant to the lymph node microenvironment in CLL.

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Declaration

Unless stated otherwise, all of the work presented in this thesis is my own work.

Abbreviations

4EBP1: eIF4E-Binding Protein 1
ADCC: Antibody Dependent Cellular Cytotoxicity
ADCP: Antibody Dependent Cellular Phagocytosis
Ag: Antigen
AID: Activation-Induced cytidine Deaminase
AKT: AKT or PKB, Protein Kinase B. 'The name AKT does not refer to its function. The "Ak" in Akt was a temporary classification name for a mouse bred and maintained that developed spontaneous thymic lymphomas. The "t" stands for 'thymoma'; the letter was added when a transforming retrovirus was isolated from the Ak strain, which was termed "Akt-8". When the oncogene encoded in this virus was discovered, it was termed v-Akt. Thus, the later identified human analogues were named accordingly' (Source: https://en.wikipedia.org/wiki/Protein_kinase_B).
AMPK: AMP-activated Protein Kinase
APE: AKT Phosphorylation Enhancer or Hook-related protein-1
APPL: Adaptor protein containing PH domain, PTB domain and Leucine zipper motif
APRIL: A Proliferation-Inducing Ligand
ArgBP2 γ : SH3 domain-containing proteins, Src and Arg-Binding Protein 2 γ
AS160: AKT substrate of 160 kDa
B: Bendamustine
BAD: BCL-2 Antagonist of cell Death
BAFF: B cell-Activating Factor of the TNF Family
BAM32: B-cell Adaptor Molecule of 32 kDa
BCR: B Cell Receptor
BIM: BCL-2 Interacting Mediator of cell death
BIRC3: Baculoviral IAP Repeat Containing 3
BMSC: Bone Marrow Stromal Cells
BR: Bendamustine (B) in combination with Rituximab (R)
BrdU: Bromodeoxyuridine
BSA: Bovine Serum Albumin
BTK: Bruton's Tyrosine Kinase
C: Cyclophosphamide
Ca²⁺: Calcium
CCD: Charge-Coupled Device
CD154: Also called CD40 ligand or CD40L
CDC: Complement Dependent Cytotoxicity
CDKis: Cyclin Dependent Kinase inhibitors
CDKs: Cyclin Dependent Kinases
CFDA-SE: Carboxyfluorescein Diacetate Succinimidyl Ester
CFSE: 6-Carboxyfluorescein Succinimidyl Ester
CFSE: Carboxyfluorescein Succinimidyl Ester (is a fluorescent cell staining dye)
c-IAP2: cellular Inhibitor of Apoptosis Protein 2
CIT: Chemoimmunotherapy
CLB: Chlorambucil
CLL: Chronic Lymphocytic Leukaemia
CpG ODN: CpG Oligonucleotides
CR: Complete Response
CRBN: Cereblon
CSR: Class Switch Recombination

CTMP: C-Terminal Modulator Protein (CTMP)
 DAG: Diacyl-Glycerol
 EBV: Epstein Barr Virus
 ECL: Enhanced Chemiluminescent
 EdU: 5-Ethynyl-2'-deoxyuridine (EdU) is a thymidine analogue
 eIF4E/G: eukaryotic translation-Initiation Factor 4E/G
 eNOS: endothelial Nitric Oxide (NO) Synthase
 ERIC: European Research Initiative on CLL
 ERKs: Extracellular-signal-Regulated Kinases
 F: Fludarabine
 FACs: Fluorescence-Activated Cell Sorting
 FBS: Fetal Bovine Serum
 FC: Fludarabine-Cyclophosphamide
 FCR: Fludarabine (F), Cyclophosphamide (C) and Rituximab (R)
 FCS: Fetal Calf Serum
 FITC: Fluorescein Isothiocyanate
 FOXO: Forkhead box class O
 FSC: Forward Scatter
 GAPs: GTPase-Activating Protein
 GC: Germinal Centre
 GCs: Glucocorticoids
 GEFs: Guanine nucleotide Exchange Factors
 GEP: Gene Expression Profiling study
 GLUT1/4: Glucose Transporter 1/4
 GPCRs: G-Protein Coupled Receptors
 GR: Glucocorticoid Receptor
 GRB10: Growth factor Receptor Binding Protein – 10
 GS: Glycogen Synthase
 GSK3: GS Kinase 3
 GTP: Guanosine Triphosphate
 Hb: Haemoglobin
 HEVs: High Endothelial Venules
 h-IL-21: human Interleukin-21
 h-IL4: human Interleukin-4
 HRP: Horseradish Peroxidase
 HSP90/CDC37: Heat Shock Protein-90 /co-chaperone CDC37
 Ig: Immunoglobulin
 IGHV: Immunoglobulin Heavy chain Variable region gene
 IGVH: Immunoglobulin Heavy chain Variable region protein
 IKZF1: Transcription factor Ikaros
 IKZF3: Transcription factor Aiolos
 ILK: Integrin-Linked Kinase
 IMiDs: Immunomodulatory Drugs
 ITAMs: Immunoreceptor Tyrosine based Activation Motifs
 JIP1: JNK Interacting Protein 1
 LDT: Lymphocyte Doubling Time
 LPS: Lipopolysaccharide
 mAbs: monoclonal Antibodies
 MBL: Monoclonal B cell Lymphocytosis
 M-CLL: CLL cells expressing Mutated IGHV gene

MDM2: Mouse Double Minute 2 homolog (MDM2) also known as E3 ubiquitin-protein ligase Mdm2 is a protein that in humans is encoded by the *MDM2* gene.

mIg: membrane Immunoglobulin

MSCs: Marrow Stromal Cells

mTOR: Mammalian Target Of Rapamycin

mTORC1/2: Mammalian Target Of Rapamycin Complex 1/2

NHL: Non-Hodgkins Lymphoma.

NK cell: Natural Killer cell

NLCs: Nurse-Like Cells

NO: Nitric Oxide

NOD/SCID γ mouse: is a strain of inbred laboratory mice, among the most immunodeficient described to date

ODN: Oligodinucleotides

OR: Overall Response

ORR: Overall Response Rate

PAGE: Polyacrylamide Gel Electrophoresis

PAK1: p21-Activated protein Kinase

PBMC: Peripheral Blood Mononuclear Cell

PBS: Phosphate-Buffered Saline, pH 7.2

PCNA: Proliferating Cell Nuclear Antigen

PDK1: PI(3,4,5)-Dependent protein Kinase-1/Phosphoinositide-Dependent protein Kinase-1

PFA: Paraformaldehyde

PFK2: 6-Phosphofructo-2-Kinase

PFS: Progression Free Survival

PH: Pleckstrin Homology

PHB2: Prohibitin 2

PHLPP: PH domain Leucine-rich repeat Protein Phosphatase

PI: Propidium Iodide

PI3K: Phosphoinositide 3-Kinase

PKA: Protein Kinase A

PKB/AKT: Protein Kinase B

PKC: Protein Kinase C

PP2A: Protein Phosphatase 2A

PTEN: Phosphatase and Tensin homologue

PVDF: Polyvinylidene Difluoride

R: Rituximab

RB: Retinoblastoma protein

RHEB: RAS Homologue Enriched in Brain

rS6: ribosomal S6 protein

RTK: Receptor Tyrosine Kinase

S1P: Sphingosine-1-Phosphate.

S6K: S6 Kinase

SDS: Sodium Dodecyl Sulphate

SHIP: SH2-containing Inositol 5-Phosphatase

SHM: Somatic Hypermutation

SSC: Side Scatter

SYK: Spleen Tyrosine Kinase

TCL1: T-Cell Leukaemia/lymphoma protein 1A

Th: T helper cells

TLR: Toll Like Receptor
TLR9: Toll Like Receptor 9
TNFR: Tumour Necrosis Factor Receptor
TRAFs: TNFR Associated Factors
TRB3: a Tribbles homolog, also known as NIPK
TSC1/2: Tuberous Sclerosis 1/2.
UK: United Kingdom
UM-CLL: CLL cells expressing Unmutated IGHV gene
US: United States
VLA-4: Very Late Antigen-4, integrin dimer, $\alpha 4\beta 1$, also known as CD49d/CD29
XIAP: X-linked Inhibitor of Apoptosis Protein
ZAP70: Zeta Associated Protein 70

Chapter 1 : Introduction

1.1 Chronic lymphocytic leukaemia (CLL)

Chronic lymphocytic leukaemia (CLL) is characterized by the accumulation of small B lymphocytes with a distinct immunophenotype in blood, bone marrow, lymph nodes or other lymphoid tissues (Zenz et al., 2010b). Most patients are asymptomatic at diagnosis. CLL can be diagnosed when the blood absolute lymphocyte count is $>5000/\mu\text{l}$. Progressive accumulation of monoclonal B lymphocytes leads to lymphocytosis (raised lymphocyte count in the blood), lymphadenopathy (swollen/enlarged lymph nodes), hepato-splenomegaly (enlargement of both the liver and spleen) and bone marrow failure (Chiorazzi et al., 2005, Zenz et al., 2010b).

CLL is defined by the presence of a monoclonal population of B cells that express simultaneously CD19, CD5, CD20 and CD23, but reduced levels of membrane IgM, IgD, and CD79b that are typically expressed by normal mature, activated B lymphocytes (Chiorazzi et al., 2005, Mulligan et al., 2009).

Monoclonal B-cell lymphocytosis (MBL) with absolute lymphocyte count below $5000/\mu\text{l}$ can occur in individuals who have no other signs of a lymphoproliferative disease. Persons with MBL displaying an immunophenotype of CLL are at risk of developing progressive CLL even if the B-cell count at presentation is low (Marti et al., 2005, Mulligan et al., 2008).

1.1.1 CLL epidemiology

1.1.1.1 United States statistics

CLL is the most common form of leukaemia in adults in Western countries. Based on 2008-2012 data from the Surveillance, Epidemiology and End Results Program which records all incidence of cancer in the United States (US), the median age at diagnosis for CLL was 71 years of age and the median age at death was 80 years of age. The number of new cases of CLL was 4.5 per 100,000 per year and the number of deaths was 1.4 per 100,000 per year. The overall 5-year relative survival for CLL in 2007 was 87.9%. It is estimated that in 2015, 14,260 men and women will be diagnosed and 4,650 men and women will die of CLL in the US.

Whilst the 5-year relative survival for CLL has increased from 67.5% in 1975 to 87.9% in 2007 (Table 1.1), the rates for both new CLL cases diagnosed and death have not changed significantly over the 10-year period from 2002 to 2012 (<http://seer.cancer.gov/statfacts/html/clyl.html>).

Table 1.1: 5-year relative survival of CLL patients in the US (1975-2007).

Year	1975	1980	1985	1990	1995	1999	2003	2007
5-year relative survival	67.5%	69.0%	74.8%	76.8%	77.9%	73.4%	81.9%	87.9%

Source: <http://seer.cancer.gov/statfacts/html/clyl.html>

The five overall survival rates are increasing with the advent of drugs like Ibrutinib. Presently, three year follow-up data of patients receiving ibrutinib has been published and it showed that the median overall survival (OS) was not reached for all patients. In patients with previously treated CLL, the 30 month OS following ibrutinib was 79% (Byrd et al., 2015).

1.1.1.2 United Kingdom statistics.

Chronic lymphocytic leukaemia is also the most common type of adult leukaemia in the United Kingdom (UK), accounting for 38% of all leukaemias. In 2011, there were 3,233 new cases of CLL; 1,957 cases being male and 1,276 female. CLL incidence is strongly related to age with an average of 43% of cases being diagnosed in those aged 65 and over in the UK between 2009 and 2011. Incidence rates are higher for males than for females from age 40-44, and this gap is widest at the age of 45-49, when the male over female incidence ratio of age-specific rates is around 25:10 (Figure 1.1). It is not known why incidence is higher in men. Indeed, prognosis is also worse in males with CLL (Catovsky et al., 2014, Molica, 2006). It has been proposed that three possible factors may contribute to the better treatment response and longer survival of women with CLL: association with good prognostic factors, pharmacokinetic differences between the sexes and finally the effect of estrogens (Catovsky et al., 2014). Out of these three factors, only the effect of estrogen may potentially also explain the differences in incidence.

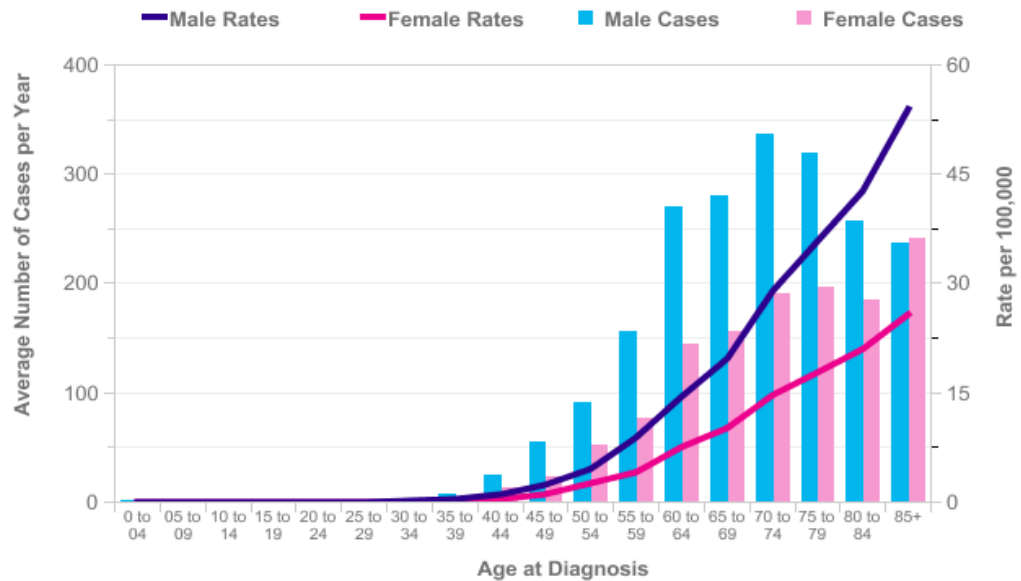


Figure 1.1: Chronic Lymphocytic Leukaemia: average number of new cases per year and age-specific incidence rates per 100,000 population, UK (2009-2011)

Source: Cancer Research UK (<http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/leukaemia-cll>)

1.1.2 Clinical staging systems

There are currently two clinical staging systems for the disease, originally devised by Drs Kanti Rai and Jacques-Louis Binet, respectively.

1.1.2.1 Rai staging

Dr Rai and colleagues proposed a method of clinical staging based on the concept that CLL is a disease of progressive accumulation of non-functioning lymphocytes (Rai et al., 1975). This method of staging proved to be a reliable predictor of survival whether used at diagnosis or during the course of the disease (Table 1.2)

Table 1.2: Rai Staging.

Stage	Clinical features at diagnosis	Median survival from diagnosis (in months)
O	bone marrow and blood lymphocytosis only	>150
I	lymphocytosis with enlarged nodes	101
II	lymphocytosis with enlarged spleen and/or liver	71
III	lymphocytosis with anemia (Hb < 11 g/dl)	19
IV	lymphocytosis with thrombocytopenia (platelets <100,000/ μ l)	19

1.1.2.2 Binet staging

A few years later, Dr Binet and colleagues published a new three-stage prognostic classification of CLL derived from a multivariate survival analysis (Binet et al., 1981). They found that thrombocytopenia and anaemia appeared to be the most important risk factors. Among the non-anaemic and non-thrombocytopenic patients, the number of involved areas of lymphoid tissues/organs was clearly related to prognosis (Table 1.3).

In 1989, the international workshop on CLL recommended integrating the Rai and Binet systems as follows: A(0), A(I), A(II); B(I), B(II); and C(III), C(IV) (iwCLL, 1989).

Table 1.3: Binet Staging.

Stage	Clinical features at diagnosis	Median survival from diagnosis (in years)
A	Blood and marrow lymphocytosis with no anaemia or thrombocytopenia and fewer than three areas* of lymphoid tissue involvement (Rai stages 0, I, and II).	>7
B	Blood and marrow lymphocytosis with three or more areas of lymphoid tissue involvement (Rai stages I and II).	<5
C	Same as B with anaemia (Hb less than 10 g/dl) and/or thrombocytopenia (platelets < 100,000/ μ l) regardless of the number of areas of lymphoid tissue enlargement (Rai stages III and IV).	<2

* Note: Areas include cervical, axillary, or inguinal lymph nodes, whether unilateral or bilateral, or spleen and liver.

1.1.3 Prognostic indicators in CLL

CLL is a heterogeneous disease. Some patients follow an indolent course of the disease, surviving over 10 years. However, other patients follow a more aggressive course with the disease progressing rapidly and these patients survive less than twelve months. There have been many attempts at understanding this heterogeneity and developing prognostic indicators in the past. Prognostic factors identified in the 1980s include level of beta-2-microglobulin and lymphocyte doubling time. Level of beta-2-microglobulin (beta-2M) in serum was found to correlate positively with clinical stage in both Rai and Binet staging systems, therefore higher levels imply a

worse prognosis (Di Giovanni et al., 1989). A long lymphocyte doubling time (LDT) (greater than 12 months) identifies patients with a very good prognosis whereas a short LDT (less than or equal to 12 months) is associated with a poorer survival and rapid disease progression (Vinolas et al., 1987, Molica and Alberti, 1987).

Today, the two most commonly used prognostic factors are chromosomal abnormalities (see [section 1.1.3.1](#)) and mutational status of immunoglobulin heavy chain variable region (*IGHV*) gene (see [section 1.1.3.2](#)). CD49d is also a robust adverse prognostic marker (see [section 1.1.3.3](#)). Research into the prognosis of certain recurrent mutations resulting in activation of oncogenes or inactivation of tumour suppression genes in CLL is currently underway (see [section 1.1.3.4](#)). Despite the discovery of several prognostic markers, currently when a patient is diagnosed with CLL only chromosomal abnormalities are routinely screened for, since they have implications on therapeutic treatment.

1.1.3.1 Chromosomal abnormalities

It was Dr Hartmut Dohner and colleagues who first proposed a hierarchical model to predict prognosis based on types of chromosomal abnormalities detected by fluorescent *in situ* hybridization (FISH) (Dohner et al., 2000). Deletions on the long arm of chromosome 13, specifically involving band 13q14, abbreviated as del (13q14), represents the most frequently observed cytogenetic aberration in CLL, occurring in 55% of all cases. In the hierarchical model, del (13q14) as the only chromosomal abnormality represents favourable prognosis (Table 1.4). Trisomy 12 has a less favourable prognosis. Deletion in 11q predicts worse prognosis. Finally, deletion in 17p represents the most unfavourable prognosis (Table 1.4).

As mentioned on page 32, the overall survival rates are increasing with the advent of drugs like ibrutinib. Three year follow-up data of patients receiving ibrutinib showed that the median overall survival (OS) at 30 months was 65% with 17p-, 85% with 11q- and 90% where neither aberration is present. In patients with previously treated CLL, the 30 month OS was 79% (Byrd et al., 2015). Only time will tell what impact drugs like these will have longer term overall survival, but they are certainly improving survival.

Table 1.4: Hierarchical model of chromosomal abnormalities in CLL

(including the incidence of chromosomal abnormalities in 325 patients with CLL)

	No of patients	% of patients	Median survival times for patients (months)
17p deletion	23	7	32
11q deletion	58	18	79
12q trisomy	53	16	114
normal karyotype	57	18	111
13q deletion as the sole abnormality	178	55	133
6q deletion	21	6	Not included in the hierarchical model
8q trisomy	16	5	
t(14q32)	12	4	
3q trisomy	9	3	

Source: (Dohner et al., 2000). Please note there are some overlaps in the groups, since the total % of patients adds up to more than 100%.

1.1.3.2 Immunoglobulin heavy chain variable region gene mutational status

At the end of the last century the correlation of prognosis with mutational status of the immunoglobulin heavy chain variable region (*IGHV*) gene was first reported by two independent groups (Hamblin et al., 1999, Damle et al., 1999). Mutation in *IGHV* refers to somatic mutation in the *IGHV* gene of CLL cells as compared with the gene sequence of the nearest germ-line, where <2% difference was classed as unmutated (UM-CLL) and $\geq 2\%$ difference was classed as mutated (M-CLL). Median survival for stage A patients with M-CLL was 293 months compared with UM-CLL which had 95 months ($P = 0.0008$). Survival was significantly worse for patients with UM-CLL irrespective of stage. UM-CLL was significantly associated with V1-69 and D3-3 usage, with atypical morphology; isolated trisomy 12, advanced stage and progressive disease (Hamblin et al., 1999). It has also been shown that patients with unmutated *IGHV* genes displayed higher percentages of CD38⁺ B-CLL cells ($\geq 30\%$) than those with mutated *IGHV* genes. Patients in both the unmutated and the $\geq 30\%$ CD38⁺ groups responded poorly to continuous multi-regimen chemotherapies

and had shorter survival (Damle et al., 1999). The exact function of CD38 is unclear; it may enhance signal transduction following the engagement of B-cell receptor (BCR) resulting in accelerated cell division and associated risk of developing genetic defects (Lanham et al., 2003). Nevertheless, it is now well established that mutated *IGHV* gene correlates with the better overall survival in patients with CLL (Figure 1.2).

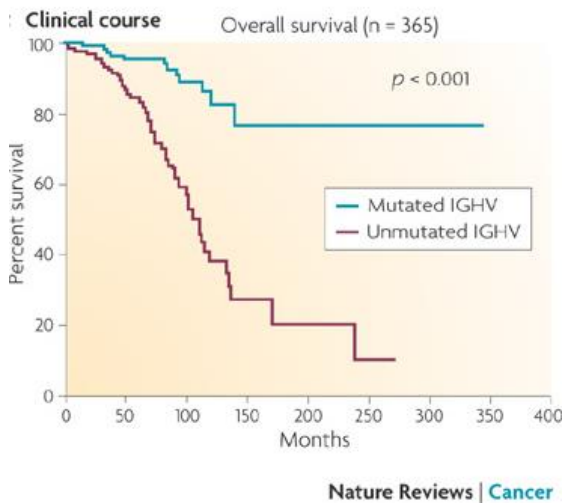


Figure 1.2: Different clinical courses associated with IGHV- unmutated and mutated CLLs.

CLL can be differentiated into those with mutated and those with unmutated immunoglobulin heavy chain variable region (IGHV) genes. These biological differences may explain the different clinical courses associated with IGHV-unmutated and mutated CLLs. In a retrospective cohort the estimated median survival was not reached for the group with mutated IGHV versus 111 months for the group with unmutated IGHV ($p < 0.001$). Figure and legend taken from figure 2c (Zenz et al., 2010b).

Following the publication of research showing that IGHV mutational status can be used to predict prognosis, there was huge interest worldwide in finding biological factors underlying the different clinical courses associated with mutated versus unmutated *IGHV* genes. These factors include different levels of protein tyrosine kinase zeta-associated protein 70 (ZAP70), CD38, different telomere lengths, altered responsiveness to BCR stimulation and a distinct likelihood of carrying or acquiring detrimental genetic lesions between the two subgroups (Table 1.5) (Zenz et al., 2010b).

Table 1.5: Two distinct subgroups of CLL based on the IGHV mutational status

UM-CLL	M-CLL
Unmutated Ig heavy chain (IGHV) gene segments	Mutated Ig heavy chain (IGHV) gene segments
Aggressive clinical course	Indolent (benign) course
Shorter survival	Longer life span
High ZAP-70	Low ZAP-70
High CD38	Low CD38
Genetically unstable.	Genetically stable.
Responsive to BCR stimulation	Less responsive to BCR stimulation

Adapted from a review by (Zenz et al., 2010b).

1.1.3.3 CD49d

Two groups in 2008 published that CD49d was a robust adverse prognostic marker (Gattei et al., 2008, Shanafelt et al., 2008). CD49d positive CLL cells had shorter treatment-free and overall survival (OS). Of note, CD49d is a good prognostic marker because the majority of cases either completely lacked expression of CD49d or expressed CD49d at very high levels, also it's easy to investigate and is highly reproducible and is not subjected to variation in fresh or frozen samples over time (Gattei et al., 2008). The prognostic relevance of CD49d was then confirmed by several other groups (Nuckel et al., 2009, Majid et al., 2011, Brachtel et al., 2011, Rossi et al., 2008), and a cut of >30% was established as CD49d positive. Many of these papers showed that CD49d expression acted as an independent prognostic marker, but that is highly associated with other risk factors such as IGHV, ZAP70, CD38 and chromosomal aberrations.

CD49d belongs to the family of integrin alpha subunits. Integrins are heterodimers of non-covalently linked alpha and beta subunits. The human CD49d (alpha 4 integrin) subunit can associate with either CD29 (beta 1 integrin) or beta 7 integrin. Very late antigen-4 (VLA-4), composed of CD49d/CD29, is the major CD49d containing combination found in resting CLL cells (Hartmann et al., 2009). VLA-4 has two major ligands: VCAM-1 expressed on endothelial cells and bone marrow cells, also the extracellular matrix molecule fibronectin. The biological function of

CD49d/CD29 integrin combination are well defined as involvement in bone marrow homing and retention of hematopoietic cells, but also in CD49d has also been shown to be involved in trans-endothelial cell migration (Till et al., 2002), adhesion and survival (including by upregulation of anti-apoptotic factors like BCL-2) (Zucchetto et al., 2012) and proliferation in CLL cells (Asslaber et al., 2013). A correlation between CD49d expression and lymphadenopathy was demonstrated (Till et al., 2002). CD49d was associated with CXCR4 expression in CLL (Majid et al., 2011).

The reader is referred to Brachtl et al.'s excellent review for further information (Brachtl et al., 2014).

1.1.3.4 Recurrent mutations in CLL

The prognostic factors such as lymphocyte doubling time, serum beta 2-microglobulin, chromosomal aberrations, *IGHV* mutational status and levels of expression in ZAP-70 and CD38 were all discovered before the end of last century. More recently, employing next generation sequencing technologies to sequence whole genomes and exomes has identified recurrent mutations in several genes in CLL cells which may have prognostic values (Wang et al., 2011, Puente et al., 2011).

These recurrently mutated genes include *TP53*, *ATM*, *MYD88*, *SF3B1*, *NOTCH1*, *DDX3X*, *ZMYM3* and *FBXW7* (Wang et al., 2011). In a second study in which 363 CLL samples were examined, four genes were found to be recurrently mutated: *NOTCH1*, *XPO1*, *MYD88* and *KLHL6* (Puente et al., 2011). Mutations in these genes correlate with some of the previously discovered prognostic markers, for example, mutations in *NOTCH1* and *XPO1* are mainly detected in patients with unmutated *IGHV*, whereas mutations in *MYD88* and *KLHL6* are mainly detected in patients with mutated *IGHV* (Puente et al., 2011).

Below is a summary of the recurrent mutations recently reported in some of the genes and their associated biological significance.

1.1.3.4.1 *TP53*

TP53 gene, located on chromosome 17p13.1, is a well-known tumour suppressor gene encoding the p53 protein. p53 acts as transcription factor which, upon DNA damage, up-regulates genes that block cell cycle progression, thereby allowing cells

to repair damaged DNA. If DNA damage is extensive, p53 can also transcribe pro-apoptotic genes, leading to apoptosis of the cells with damaged DNA, as reviewed in (Wickremasinghe et al., 2011). p53 also has transcription-independent functions and is capable of activating the mitochondrial apoptosis pathway by interacting with the core apoptosis-regulatory BCL-2 family of proteins located on the mitochondrial outer membrane. For example, it has been shown that direct interaction of p53 with BCL-2 resulting in conformational change of Bax and activation of mitochondrial apoptosis is the major mechanism of apoptosis induction by chlorambucil, fludarabine and the MDM2 inhibitor nutlin-3a in CLL cells (Steele et al., 2008).

In CLL *TP53* is recurrently deleted (through deletion of chromosome 17p) in about 5-15% of patients at diagnosis (Dohner et al., 1995, Dohner et al., 2000) and has long been known to associate with adverse clinical outcome and drug resistance. Most CLL patients with monoallelic 17p deletion also carry *TP53* mutations in the remaining allele (>80%) (Zenz et al., 2008b, Rossi et al., 2009, Dicker et al., 2009, Malcikova et al., 2009). FISH can quantify the clone size of cells with 17p deletion. Consequently, whilst patients with monoallelic 17p deletion may initially respond to chemotherapy (p53-mediated treatment) they all eventually relapse, as clonal selection occurs to leave only the *TP53* mutated/17p deleted cells which are resistant and thus these patients have a very poor prognosis (Catovsky et al., 2007).

Recently, *TP53* mutations have been detected in a proportion (between 3-8.5%) of CLL patients who do not carry 17p deletion and this is associated with poor clinical outcome (Zenz et al., 2008b, Zenz et al., 2010a, Gonzalez et al., 2011, Rossi et al., 2009, Dicker et al., 2009, Malcikova et al., 2009).

After treatment with chemotherapy (purine analogues), patients who are refractory are often observed to have *TP53* mutations or deletions (Malcikova et al., 2009). Whilst only a small amount of patients (5-10%) have a *TP53* abnormalities at diagnosis, this may reach up to 40-50% in refractory disease (Rossi et al., 2009, Zenz et al., 2010a, Gonzalez et al., 2011, Zainuddin et al., 2011). This is most likely due to clonal selection rather than being induced by therapeutic agents.

Given the importance of the p53 pathway in tumour suppression, there are other known ways in which the function of *TP53* can be affected other than through *TP53*

mutation and deletion, such as ATM inactivation. Functional impairment of the p53 pathway because of inactivation of *TP53* or *ATM* is readily detectable using a simple assay, in which CLL cells are exposed to ionizing radiation, cultured overnight and levels of p53 and p21 (a transcriptional target of p53) measured by Western blotting or flow cytometry (Pettitt et al., 2001, Carter et al., 2004). Using this approach, levels of p53 itself determine the type of dysfunction and levels of up-regulation of p21 in response to ionizing radiation defines the state of p53 dysfunction. Type A defect is associated with *TP53* mutation, therefore baseline p53 levels are increased, since MDM2 is unable to degrade mutant TP53 protein. Type B defect is associated with *ATM* mutation, baseline p53 levels are not increased, but there is impaired accumulation of p53 in response to ionizing radiation (Pettitt et al., 2001). Both types of p53 dysfunction are strongly associated with short survival (Lin et al., 2002). A third, type C defect, is characterized by failure of p21 protein accumulation despite a normal p53 protein response and was found amongst more clinically aggressive cases (Johnson et al., 2009).

Functionally, mutations in the DNA-binding motif of p53 are closely associated with poor survival in CLL (Trbusek et al., 2011). In addition, patients carrying small *TP53*-mutated sub-clones (less than 20%) have the same unfavourable prognostic impact as those of patients containing clonal *TP53* defects such as 17p deletion, since over time these small sub-clones expand to become the predominant population in CLL (Rossi et al., 2014). Direct Sanger sequencing of exons 4-9 of *TP53* gene is thus recommended as the best test to identify *TP53* mutations (Pospisilova et al., 2012). The topic of *TP53* mutation in CLL was well reviewed by a recently by Malcikova and colleagues (Malcikova et al., 2014).

1.1.3.4.2 ATM

The *ATM* gene, with 62 coding exons, is located on chromosome 11q23. ATM is a protein kinase that, following DNA double strand breaks, activates p53 and coordinates cellular responses, including DNA repair, induction of cell cycle arrest and activation of apoptosis. As described earlier, chromosomal deletion in 11q is relatively common, occurring in ~18% of all CLL patients (Dohner et al., 2000). In addition, 36% of CLL patients with chromosomal 11q deletion have mutations in the remaining *ATM* allele and their prospect of survival is significantly worse than those

patients with 11q deletion alone ($p=0.0283$) (Austen et al., 2007). This finding was further confirmed in a prospective randomised trial where patients with both *ATM* mutation and 11q deletion had significantly reduced progression-free and overall survival following front-line therapy with alkylating agents and purine analogues (Skowronska et al., 2012). In fact, the progression free survival of patients with biallelic *ATM* activation was similar to that of patients with *TP53* loss and/or mutation. Further information on the biological functions of *ATM* and consequences of its loss in disease progression of CLL including the distinct clinical behaviour resulting from monoallelic and biallelic *ATM* alterations, as well as assays for the detection of *ATM* inactivation, has been eloquently summarised in a recent review (Stankovic and Skowronska, 2014).

1.1.3.4.3 *NOTCH1*

The NOTCH signalling pathway plays an important role in the development and function of lymphocytes (Radtke and Raj, 2003, Pear and Radtke, 2003, Lobry et al., 2014). Both NOTCH1 and NOTCH2 have been found to be constitutively active in CLL cells, but not in normal B cells, and to contribute to the survival and apoptosis resistance of CLL cells (Rosati et al., 2009, Osborne, 2009). Gain-of-function mutations in *NOTCH1* gene have been detected in ~10% CLL patients at diagnosis and their presence is associated with a more advanced clinical stage of the disease and significantly shorter overall survival (Puente et al., 2011, Fabbri et al., 2011, Rossi et al., 2012b, Oscier et al., 2013). Also, *NOTCH1* mutations are commonly found in CLL patients with unmutated *IGHV* and/or trisomy 12 (Oscier et al., 2013, Puente et al., 2011). *NOTCH1* mutations, whilst mutually exclusive with *TP53* mutations, have a similar prognostic value to *TP53* mutations and can independently predict poor overall survival in CLL patients (Rossi et al., 2012b). *NOTCH1* mutations (in exon 34) were identified as an independent prognostic marker in a prospective clinical trial (UK LRFCLL4 trial) (Oscier et al., 2013).

1.1.3.4.4 *SF3B1*

SF3B1 is a critical component of the RNA splicing machinery that ensures successful transcription (Wan and Wu, 2013). *SF3B1* mutations were discovered by whole genome sequencing in 9.7% of individuals with CLL and were commonly found in patients with unmutated *IGHV* gene (Quesada et al., 2012). A separate study

using parallel sequencing of 88 whole exomes and whole genomes identified *SF3B1* as the second most frequently mutated gene occurring in 15% of patients (Wang et al., 2011). *SF3B1* mutations were also found primarily in patients with 11q deletion in this study. It has been shown that over time, representation of CLL clones carrying *SF3B1* mutations increases independently of treatment or *TP53* mutations, suggesting that clones with *SF3B1* mutations either grow faster or die slower than other clones, leading to clonal expansion (Schwaederle et al., 2013). In a prospective clinical study (UK LRF CLL4), *SF3B1* mutations (exon 14-16) were found in 17% of patients and significantly associated with high CD38 expression and shorter overall survival (Oscier et al., 2013). *SF3B1* mutations were identified as an independent prognostic marker for poor overall survival in the same study.

Traditionally the view was that *SF3B1* mutations might lead to aberrant splicing of specific transcripts that effect the pathogenesis of CLL, however no candidate aberrantly spliced gene could be functionally linked to CLL pathogenesis of chemo resistance. A novel splice junction in the *ATM* gene in *SF3B1* mutated cases may explain the observed functional overlap between *SF3B1* and *ATM* mutations in CLL (Ferreira et al., 2014), however although Te Raa and colleagues, were able to detect it, it was present at such low levels that they felt it did not explain the defects observed in CLL cells (Te Raa et al., 2015). Strikingly though, they found that *SF3B1* mutated samples (without concurrent *ATM* or *TP53* aberrations) displayed partially defective ATM/p53 transcriptional and apoptotic responses. They measured the transcription of six target genes by RT-MPLA: CD95, p21, Bax, Puma, FDXR and PCNA after irradiation or fludarabine and doxorubicin; and also as measured at protein levels of p21 and puma and levels of cell death. In sole mutated *SF3B1* cases, DNA damage was increased at baseline and upon irradiation. *SF3B1* mutations therefore are associated with increased DNA damage or aberrant response to DNA damage. Te Raa and colleagues suggest that further research is required to explore the mechanism and causality of the link between *SF3B1* mutations and DNA damage response (Te Raa et al., 2015). Larrayoz and colleagues suggested that targeting the spliceosome might therefore have benefit. They showed that *SF3B1* inhibition, by spliceostatin A (SSA) was able to induce more apoptosis in CLL cells than normal B cells, but that this occurred irrespective *SF3B1* mutational status. They showed that the SSA-induced apoptosis correlated with altered MCL1 splicing and

downregulation of MCL1 protein, suggesting that cell death is regulated by altered MCL1 splicing (Larrayoz et al., 2016).

1.1.3.4.5 *BIRC3*

The gene *BIRC3* (baculoviral IAP repeat containing 3) is located on chromosome 11q22 and encodes a cellular inhibitor of apoptosis protein 2 (c-IAP2) (Liston et al., 1996). Among its many biological functions, c-IAP2 is a negative regulator of the non-canonical NF- κ B signalling pathway (Zarnegar et al., 2008, Gardam et al., 2011). Loss-of-function mutations in *BIRC3* gene in CLL cells, first reported by Fabbri and colleagues (Fabbri et al., 2011), results in truncated c-IAP2 protein, which subsequently leads to activation of non-canonical NF- κ B signalling and enhanced survival of leukaemic clones. Of interest, *BIRC3* mutations are rare in CLL at diagnosis (4%), but present in 24% of fludarabine-refractory CLL cases, suggesting that *BIRC3* mutations may be specifically associated with the fludarabine-refractory phenotype possibly caused by increased NF κ B activity (Rossi et al., 2012a). The European Research Initiative on CLL (ERIC) found *BIRC3* mutations associated with UM-CLL, del(11q) and trisomy 12 (Baliakas et al., 2015). Finally, it is worth mentioning that *BIRC3* disruption (mutation and/or deletion) occurs in an almost mutually exclusive manner with *TP53* mutation and/or deletion, *NOTCH1* mutations or *SF3B1* mutations (Rossi et al., 2012a).

1.1.3.4.6 Other recurrent mutations

Other genes that have been reported to be recurrently mutated in CLL cells at different stages of the disease include *MYD88*, *XPO1*, *KLHL6*, *ZMYM3*, *MAPK1*, *FBXW7*, *DDX3X* (Wang et al., 2011, Puente et al., 2011).

The functional significance and clinical relevance of many of these mutated genes have been extensively reviewed elsewhere (Martinez-Trillos et al., 2013) and will not be repeated here.

One emerging feature from these studies reporting recurrent gene mutations is that CLL is an extremely heterogeneous disease in which only a few genes are mutated in 10-15% of patients, whilst a large number of genes are mutated at lower frequencies (2-5%). However, with whole genome and exome sequencing, it is possible to obtain high sequencing depth which enables reliable detection of sub-clonal mutations that

define sub-clones, tracking them over time and linking sub-clonal mutations to clinical outcome. Indeed, it has been shown that some sub-clones with driver mutations in genes such as *SF3B1* and *TP53* can expand over time and become dominant clones in the phase of rapid disease progression (Landau et al., 2013).

1.1.3.5 A new prognostic score for CLL

Over the years many new prognostic markers have been discovered in CLL with most discovered in retrospective studies without taking other markers into consideration. The German CLL study group (GCLLSG) used large prospective clinical trial cohorts to propose a new prognostic score, which uses eight factors to infer survival from study entry (Pflug et al., 2014). This new prognostic score, which does not include Rai or Binet staging reflects, the evolution of our understanding of the disease in recent years. The score determines 17p deletion, unmutated *IGHV* and 11q deletion as having prognostic significance, as well as serum markers for proliferation as measured by thymidine kinase and β_2 -microglobulin. Finally host factors such as age, gender and performance (fitness) status have been taken into account. This new prognostic score has not yet been adopted into clinical practice for several reasons. For example, not all clinical laboratories can perform assays to measure serum thymidine kinase activity or p53 mutations. In addition, information on novel mutations on genes such as *NOTCH1*, *SF3B1* and *BIRC3* has not been considered. Finally, with the recent development of small molecule inhibitors targeting BCR signalling pathways, the applicability of this prognostic score will need re-evaluation in light of the introduction of these novel, highly effective non-chemotherapeutic agents into the clinic (Tam and Seymour, 2014).

1.1.4 Pathogenesis of CLL

As described earlier, CLL is a heterogeneous disease. This heterogeneity can be manifested by genetic alterations, distinct clinical courses and different responses to treatment in individual patients. It is therefore important to know how CLL initially develops and what factors influence the progression of the disease.

1.1.4.1 Cell origins of CLL

The cell origins of CLL are still a subject of debate. Based on the evidence from studies on *IGHV* mutational status and gene usage, gene expression profiling and DNA methylome in CLL, a consensus is emerging that a different cell of origin exists for M-CLL versus UM-CLL (Zhang and Kipps, 2014, Gaidano et al., 2012, Chiorazzi and Ferrarini, 2011, Seifert et al., 2012).

1.1.4.1.1 IGHV mutational status and gene usage

During normal B cell development, immature B cells egress from the bone marrow and complete their maturation in the periphery, where they differentiate into various B cell types dependent upon the microenvironmental influences (Rickert, 2013). When a B cell encounters an antigen via its BCR, it enters the germinal centre (GC) of a lymph node, where it interacts with CD4+ T helper cells, also known as T follicular helper (Tfh) cells. In the presence of other co-stimuli, the B cell proliferates in the GC. To increase the affinity of the BCR for the antigen, the B cell also undergoes point mutations in the V gene segments of the immunoglobulin heavy chains (*IGHV*) by somatic hypermutation (SHM) and class-switch recombination (CSR) driven by activation-induced cytidine deaminase (AID) (Rickert, 2013).

As described earlier, patients with CLL can be divided into two sub-groups based on mutational status of *IGHV* genes. The cell origin of CLL in cases with mutated *IGHV* is generally assumed to come from antigen-driven and GC dependent memory B cells, whereas CLL cells with unmutated *IGHV* are generally considered to derive from antigen-naïve B cells (Seifert et al., 2012).

Furthermore, both *IGHV*-mutated and *IGHV*-unmutated CLL cells have “stereotyped” *IGVH* genes, and display a restricted repertoire of V, D and J segments, thus inferring a role for antigenic stimulation in CLL (Fais et al., 1998, Tobin et al., 2004, Agathangelidis et al., 2012, Messmer et al., 2004a, Murray et al., 2008).

The specific antigens that drive CLL have not been identified, but they may be of self-origin (such as myosin heavy chain IIA) (Lanemo Myhrinder et al., 2008, Catera et al., 2008, Chu et al., 2010, Chu et al., 2008, Binder et al., 2010), viral (such as CMV) (Steininger et al., 2012, Kostareli et al., 2009, Steininger et al., 2009),

bacterial (Binder et al., 2003, Hatzi, 2006, Landgren et al., 2007), or fungal origin (Hoogeboom et al., 2013, Binder et al., 2013, Greaves, 2013). More on the topic of inferring the role of antigen in CLL can be found in articles by Kostareli and colleagues and Rosen and colleagues (Kostareli et al., 2012, Rosen et al., 2010).

Self-origin antigens may include myosin heavy chain IIA which is expressed by apoptotic cells, or lipid oxidation products created during apoptosis or vimentin (Lanemo Myhrinder et al., 2008, CATERA et al., 2008, Chu et al., 2010, Chu et al., 2008, Binder et al., 2010). Further evidence for an autoantigen was provided by Zwick and colleagues who used human tissue-derived protein macroarrays to show that autoantigens could be identified for 25.5% of the cases tested (Zwick et al., 2013).

Viral infections have also been suggested to drive subgroups of CLL cases. A small number of UM-CLL IGHV 1-69 encoded BCRs react with a cytomegalovirus phosphoprotein antigen (Steininger et al., 2012) and many CLL patients have had persistent EBV or CMV infections (Kostareli et al., 2009, Steininger et al., 2009).

CLL mAbs have been shown to react against various bacterial antigens: for example capsular polysaccharides and oxidized LDL (oxLDL) from *Streptococcus pneumoniae* (Binder et al., 2003), as well as antigens on *Streptococcus pyogenes*, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterobacter cloacae* (Hatzi, 2006). An epidemiologic study showed that recurrent respiratory tract infections caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* were associated with an increased risk of CLL (Landgren et al., 2007).

A subgroup of mutated CLL patients (VH7-3) have specificity for β -(1,6) glucan cell wall constituents of commensal yeast and filamentous fungi (Hoogeboom et al., 2013, Greaves, 2013). These CLL cells can bind to and proliferate in response to β -(1,6) glucan.

Alternatively, the BCRs from CLL cells may be capable of antigen-independent signalling. Intermolecular interactions between conserved residues in the framework region of the variable heavy-chain gene segment and the antigen-binding sites in selected variable heavy chain complementarity-determining region 3 (CDR3) sequences result in BCR auto-crosslinking and antigen-independent BCR signalling,

as measured by calcium signalling (Duhren-von Minden et al., 2012). However, Hoozeboom and colleagues argue that this does not explain why there are subgroups of CLL patients expressing highly similar BCRs with shared somatic mutations (Hoozeboom et al., 2013). To reconcile the difference and accommodate the idea of autonomous antigen-independent signalling, it has been suggested that most ligands could be considered to be autoantigens since stereotyped BCRs exist in unrelated patients from different parts of the world (Chiorazzi and Efremov, 2013). Also, there is always some ongoing stimulation of CLL *in vivo*, as shown by phosphorylation of signalling kinases. Autonomous BCR signalling seems to be a feature of CLL.

Binder and colleagues have also presented data that suggests an autostimulatory mechanism in CLL since the FR2 domain of human IGVH appears to interact with the HCDR3s of CLL cells (Hoozeboom et al., 2013, Binder et al., 2013). This could be argued to be an exemplary autoreactivity (Chiorazzi and Efremov, 2013).

It has been suggested that BCR autonomous antigen-independent signalling and a fungal driver are not incompatible theories, as it is possible that CLL cells originate as antigen-dependent but evolve to become more autonomous, or the other way around (Greaves, 2013).

1.1.4.1.2 Gene expression profiling

Despite the difference in prognosis between M-CLL and UM-CLL, gene expression profiling (GEP) studies showed that, among 12,000 genes identified, there were only 23 genes that were differentially expressed between M-CLL and UM-CLL, and that CLL cells from the two sub-groups were more similar to memory B cells than naïve B cells (Klein et al., 2001, Rosenwald et al., 2001).

A more recent GEP study indicated that *IGHV* unmutated CLL cells tend to be more similar to pre-GC CD5⁺CD27⁻ B cells whereas mutated CLL cells show a higher similarity to post-GC CD5⁺CD27⁺ B cells (Seifert et al., 2012). The authors showed that the stereotyped *IGHV* rearrangements that are commonly seen in CLL are predominantly found in mature CD5⁺ B cells from healthy donors, thus suggesting a CD5⁺ B cell origin of CLL.

1.1.4.1.3 DNA methylome of CLL

Whole genome analysis of the DNA methylome of CLL cells has shown that M-CLL and UM-CLL retain an epigenetic imprint of memory B cells and CD5⁺ naïve B cells respectively (Kulis et al., 2012). Of note, the same study also identified a distinct third subgroup termed intermediate IGHV mutated CLL (i-CLL) which represents about 15% of all cases studied. Although the cell origin of i-CLL is unknown, it is postulated that it may derive from a GC-independent marginal zone B cell that has undergone low levels of somatic hypermutation after experiencing an antigen or antigens. A similar study later revealed very different clinico-biological features and outcome of CLL patients from the three subgroups. Applying time to first treatment and overall survival as end points, it has been shown that naïve B cell-like CLL (UM-CLL) is clinically adverse, memory B cell-like CLL (M-CLL) is clinically favourable and that i-CLL displays a clinical behaviour in between the former two subgroups (Queiros et al., 2015). The study also showed that i-CLL exhibits a significant increase of *SF3B1* mutations (22% vs 9% in naïve B cell-like CLL and 4% in memory B cell-like CLL). However, the exact role of the mutation in the pathogenesis of i-CLL is unclear.

Therefore, based on gene transcriptional and epigenetic data, it is most likely that CLL cells originate from CD5⁺ B cells at different maturation stages with pre-GC counterparts leading to UM-CLL and post-GC B cells causing M-CLL.

1.1.4.1.4 Stem cell origin

A recent study using xenotransplanted mice suggests that the cell of intrinsic abnormality is at the level of haematopoietic stem cells (HSC) (Kikushige et al., 2011). However none of the known chromosomal abnormalities found in CLL were discovered in the HSC compartment. HSCs obtained from patients with CLL were transplanted into mice and they produced B cells with restricted mono- or oligo-clones with CLL-like phenotype. However, these B cell clones were independent of the original CLL clones because they expressed their own immunoglobulin VDJ genes. Since these cells had none of the abnormal karyotypes, they might be better designated as MBL-like clones which require further accumulation of genetic alterations to transform into clinical CLL.

In conclusion, as summarised in Figure 1.3, the emerging consensus is that M-CLL cells appear to undergo GC- and T cell-dependent differentiation whereas UM-CLL cells experience antigen independently of T cells. However, the jury is still out regarding cell origins of CLL.

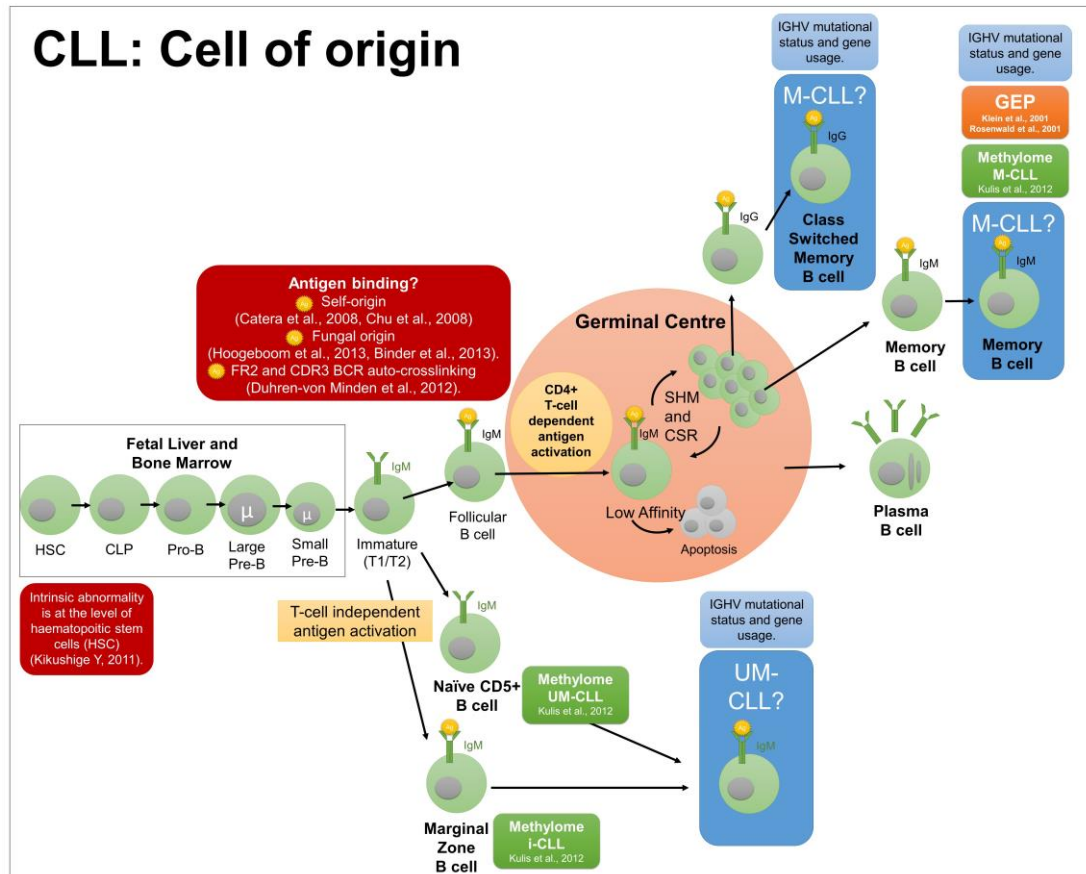


Figure 1.3: CLL cell of origin

B cells all originate from haematopoietic stem cells (HSC) which give rise to common lymphoid progenitors (CLP), then pro-B cells, followed by pre-B cells which express cytoplasmic μ H chains in fetal liver and bone marrow. Immature B cells are also referred to as “transitional” (T1 and T2) based on their phenotypes and ontogeny. Immature B cells respond to T cell-independent type 1 antigens such as LPS, which elicit rapid responses in the absence of MHC class II-restricted T-cell help. These cells express unmutated BCRs. B cells can undergo CD4+ T cell dependent antigen-activation in the germinal centre (GC). GC-derived memory B cells express mutated (and sometimes class-switched) BCRs with enhanced affinities for the antigen, the product of somatic hypermutation (SHM) and even class switch recombination (CSR). Memory B cells can exist after antigen challenge and can terminally differentiate into antibody secreting plasma cells. There is evidence that there is an intrinsic abnormality in HSC of CLL patients. Based on IGHV status and gene usage, generally M-CLL cells are considered to be antigen-GC-experienced B cells and UM-CLL cells are generally considered have undergone T-cell independent antigen activation, and be of marginal zone B cell origin. Methyloyme data suggests that M-CLL cells are memory CD5+ B cells, UM-CLL cells are naïve CD5+ B cells and intermediate-CLL cells are CD5+ marginal zone B cells. Gene expression profiling (GEP) studies show that CLL cells resemble memory CD5+ B cells irrespective of mutational status. Figure adapted and based on reviews (LeBien and Tedder, 2008, Rickert, 2013, Zhang and Kipps, 2014).

1.1.4.2 The CLL microenvironment

Understanding the microenvironment in CLL is important since CLL cells not only proliferate in lymph nodes, but they also survive better and become resistant to chemotherapeutic agents in these niches, leading to minimal residual disease in these locations (Meads et al., 2009). Indeed, it has been found that culturing CLL cells with accessory cells or cytokines can rescue them from spontaneous apoptosis (Burger, 2011, Caligaris-Cappio et al., 2014). It is crucial that any new therapeutic agents target not only non-cycling CLL cells in the peripheral blood, but also those residing in the tissue microenvironment (Bogner et al., 2003).

It is well known that CLL pathogenesis is strongly influenced by the tissue microenvironment (Burger, 2011, Caligaris-Cappio et al., 2014). CLL cells interact with many cells: T cells, bone marrow stromal cells (BMSC), monocyte-derived nurse-like cells (NLC), and endothelial cells (EC) as well as antigens leading to BCR activation resulting in CLL-cell survival, proliferation and drug resistance, as described in Figure 1.4.

The proliferation centres in CLL lymph nodes are the niches where NF- κ B activation takes place, leading to CLL survival, proliferation and inhibition of apoptosis. It is plausible that the process is triggered by concerted signaling from BCR, CD40 and TNF receptors (such as BAFF), with the help of T cells and specific subpopulations of macrophages (Herrerros et al., 2010).

The following sections will review the interactions between CLL cells and antigens via BCR, T cells, BMSCs and NLCs, focusing particularly on T cell-CLL cell interactions via the CD154-CD40 signalling pathway.

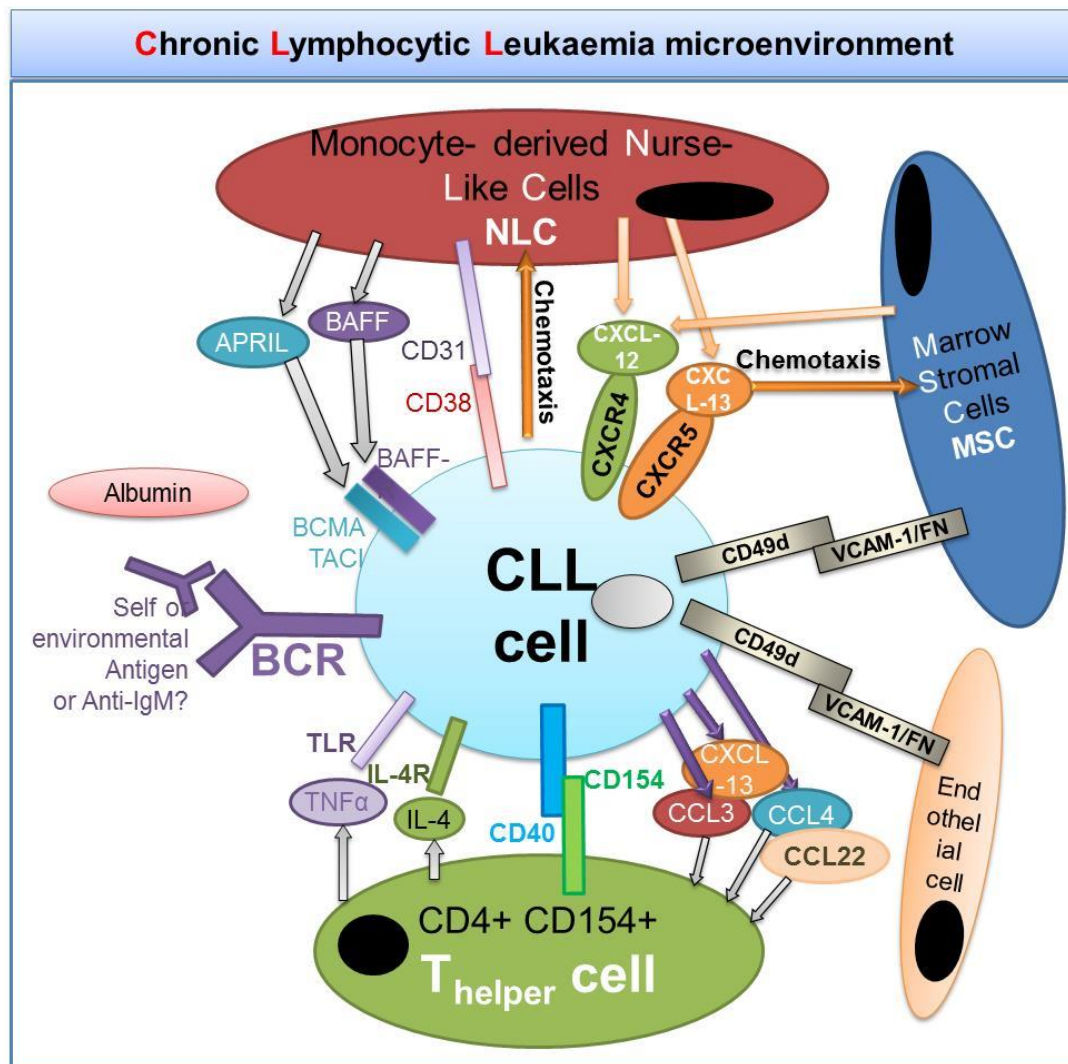


Figure 1.4: The Chronic Lymphocytic Leukaemia microenvironment

Molecular interactions between CLL and stromal cells in the BM and/or lymphoid tissue microenvironments that are considered important for CLL-cell survival and proliferation. Contact between CLL cells and monocyte-derived Nurse-Like Cells (NLCs) or Marrow Stromal Cells (MSCs) are established and maintained by chemokine receptors and adhesion molecules expressed on CLL cells. NLCs express the chemokines CXCL12 and CXCL13, whereas MSCs predominantly express CXCL12. NLCs and MSCs attract CLL cells via the G protein-coupled chemokine receptors CXCR4 and CXCR5, which are expressed at high levels on CLL cells. Integrins, particularly VLA-4 integrins (CD49d), expressed on the surface of CLL cells establish cell-cell adhesion through VCAM-1 and fibronectin on endothelial cells and marrow stromal cells. NLCs also express the TNF family members B cell-activating factor of the TNF family (BAFF) and A Proliferation-Inducing Ligand (APRIL), providing survival signals to CLL cells via corresponding receptors (BCMA, TACI, and BAFF-R). CD38 expression allows CLL cells to interact with CD31, the ligand for CD38 that is expressed by stromal and NLCs. Self- and/or environmental Ags are considered key factors in the activation and expansion of the CLL clone by activation of the BCR and its downstream kinases. This can be mimicked using Anti-IgM in vitro assays. CD154+ T cells interact with CLL cells via CD40. T cells also release IL-4 and TNFα which signal via IL-4R and TLR on CLL cells. BCR stimulation or co-culture with NLCs induces CLL cells to secrete chemokines (CCL3, CCL4, and CCL22) for the recruitment of T cells. Figure adapted from the review (Burger, 2011).

1.1.4.2.1 T cells interact with CLL cells

It has been known for some time that CLL cells intermingle with CD4⁺ T helper cells in pseudofollicles of lymph nodes (Schmid and Isaacson, 1994) and bone marrow (Pizzolo et al., 1983). The close contact between activated CD4⁺ T cells and CLL cells results in upregulation of CD38 expression in CLL cells, which, as a result, are primed to proliferate (Patten et al., 2008). This proximity in locality is not a coincidental event as CLL B cells were shown to be able to attract CD4⁺, CD154⁺ T cells by producing CCL22 (Ghia et al., 2002).

The need for T cells in the progression of CLL was most clearly demonstrated in the adoptive-transfer model, as described below. When human CLL cells were transplanted into the immunodeficient NOD/SCID γ mouse in the absence of T helper (Th) cells, the CLL cells failed to expand in the host. When autologous T cells were co-transferred into the mouse along with the CLL cells, the leukaemic cells were able to survive and proliferate, and they recapitulated key features of human CLL cells in aspects of growth kinetics, CD38 expression, and accumulation in secondary lymphoid tissues (Bagnara et al., 2011).

It has been increasingly recognised that proliferation of CLL cells in lymph nodes and bone marrow is driven by cognate interactions with Th cells, and CD40-CD154 interaction is one of the most important mechanisms for T-cell mediated CLL-cell survival and proliferation. CD154 is expressed by CD4⁺ Th cells in lymph nodes and bone marrow (Granziero, 2001).

Tretter and colleagues have shown that co-culture of CLL cells with activated T cells resulted in improved survival and proliferation of the leukaemic cells *in vitro* (Tretter et al., 1998). Addition of CD40 antibody partially inhibited activation of CLL cells, implicating an involvement of CD40-CD154 interaction in the proliferative response of CLL cells to T cells, although other signals are also required. (Tretter et al., 1998).

Recently Os and colleagues showed that activation of CLL cells by Th cells was dependent on cell-cell contact and involved CD154 (Os et al., 2013). Th cells supported CLL-cell activation and proliferation *in vitro*, which was inhibited by addition of anti-CD154 antibody. Using a xenograft model similar to the adoptive

transfer model, they showed that Th cells also supported CLL-cell proliferation in the bone marrow of NOD/SCID γ mice (Os et al., 2013).

Whilst Patten's group was 'unable to demonstrate CD40L expression in tissue sections' of both the cryopreserved and paraffin-embedded CLL lymph node sections by multi-parameter confocal immunofluorescence microscopy. They suggested that this was 'presumably because of technical factors and because, as previously reported (Cantwell et al., 1997), expression levels may be relatively low because of receptor-mediated down-regulation induced by contact with CD40 on the tumour surface' (Patten et al., 2008). Jaksic and colleagues, agreed with Patten about the expression of CD40L (CD154) on T cells, they found CD154 was absent/low on T-cells and absent/low in 14/21 patients on B-CLL cells. However in 7/21 patients B-CLL cells had higher CD154 expression and CD154 was most expressed in B-CLL cells in lymph nodes. They showed that all B-CLL cells expressed CD40 (Jaksic O, 2010). Other groups, such as Ghia and colleagues have shown contact between CD40L-expressing T cells and Ki-67 CLL cells (Ghia et al., 2002), and more recently Herreros and colleagues showed that proliferation centres in CLL patients had more T cells (CD3-positive cells) than surrounding tissue, many of which expressed CD40L, as shown by double immunofluorescence (Herreros et al., 2010).

1.1.4.2.1.1 CD40-CD154 interaction

CD40 is a member of the tumour necrosis factor receptor (TNFR) family, and is expressed on B cells. CD40 and its cognate ligand, CD154, are expressed on many types of immune cells. Herein we shall consider only the CD40-CD154 interaction between T and B cells. This interaction is important for B cell survival and proliferation, as well as germinal centre (GC) formation, immunoglobulin (Ig) isotype switching, somatic hypermutation (SHM) of the Ig to enhance affinity for antigen, and the formation of long lived plasma cells and memory B cells (Elgueta et al., 2009, van Kooten and Banchereau, 2000, Rickert et al., 2011).

Aside from T cells, CD40 stimulation may occur from soluble CD40L, since it has been shown that the serum of patients with CLL contained elevated levels of biologically active soluble CD40L, and that CD40L can prolong survival of CLL cells *in vitro* (Younes et al., 1998).

1.1.4.2.1.2 CD40 signalling.

In order to understand the CD40-CD154 interactions in human B-lymphocytes, many *in vitro* models have been developed including the use of monoclonal antibodies (mAb), recombinant proteins, anti-CD40 mAb immobilized on CD32+ L cells, or even CD154+ cells. All of these models have different intensity of CD40 stimulation, probably due to differences in CD40 oligomerization (Neron et al., 2011).

CD154-CD40 interaction is generally thought to occur in a trimerised fashion (McWhirter et al., 1999), although higher orders of oligomerisation are possibly required for full activation (Haswell et al., 2001, Pullen et al., 1999, Barr and Heath, 2001). Whilst there will be differences in the signalling in the different cell types that express CD40, broadly speaking engagement of CD40 by CD154 results in the recruitment of adapter proteins known as TNFR-associated factors (TRAFs) to the cytoplasmic domain of the CD40 receptor (Figure 1.5). TRAF1, 2, 3, 5 and 6 can all be recruited to the intracellular tail of CD40 (Bishop et al., 2007). The exact signalling pathways following CD40 stimulation are still being elucidated in different cell types (Elgueta et al., 2009, Rickert et al., 2011). It may be that the order of CD40 oligomerization affects the ability of these TRAFs to bind the CD40 cytoplasmic domain.

In B cells and in monocyte-derived dendritic cells, upon binding to CD40, TRAFs activate different downstream signalling pathways, including the canonical and non-canonical NFκB signalling pathway, the MAPK, PI3K and PLCγ signalling pathways (Bishop et al., 2007). JAK3 has been shown to bind directly to CD40, in and monocyte-derived dendritic cells results in STAT5 phosphorylation (Saemann et al., 2003, Saemann et al., 2002), however in B cells JAK3 was not seen to be phosphorylated (Hanissian and Geha, 1997, Revy et al., 1999).

TRAF6 binds to the proximal amino acid sequence QxPxEx of CD40, and activates TAK1, which promotes activation of the NFκB canonical pathway (Bishop et al., 2007, Lomaga et al., 1999, Jalukar et al., 2000). TRAF6 also activates PI3K, p38 and JNK signalling pathways (Rowland et al., 2007, Davies et al., 2005, Benson et al., 2006). TRAF6 may also have a functional role in CD40 signaling, without binding directly to CD40, but possibly by interacting with TRAF2 activate JNK (Rowland et al., 2007).

In dendritic cells (DCs) and osteoclasts, CD40 can induce the recruitment of TRAF6/Cbl-b/PI3K complex, which leads to AKT phosphorylation (Arron et al., 2001, Davies et al., 2004). Indeed, TRAF6 is known as been shown to recruit AKT to the plasma membrane following IGF-1 stimulation, and so it is not impossible that TRAF6 is working in this manner in this system too (Yang et al., 2009).

TRAF2 and TRAF3 bind to the same distal region, demarcated by the amino acid sequence PxQxT (Manning et al., 2002a). CD40-induced activation of TRAF2 induces c-IAP1 proteins to ubiquitylate TRAF3, promoting its degradation and releasing MEKK1 and stable expression of autocatalytic NIK for downstream signalling, leading to activation of the non-canonical NFκB pathway. TRAF2 and TRAF3 also activate p38 and JNK and AKT signalling pathways (Brown et al., 2002, Gardam et al., 2008, He et al., 2007, Hostager et al., 2003, Lee et al., 1997, Vallabhapurapu et al., 2008, Xie et al., 2004, Yeh et al., 1997, Zarnegar et al., 2008).

TRAF5 associations with CD40 are indirect via TRAF3 (Bishop et al., 2007), leading to proliferation, costimulatory molecule expression and antibody production (Hauer et al., 2005, Nakano et al., 1999).

Little is known about TRAF1 in CD40 signalling, despite its expression being heightened during CD40 stimulation (Zapata et al., 2000, Schwenzer et al., 1999). In the absence of TRAF2, TRAF1 can bind the distal TRAF2-binding site, but in normal circumstances it does not appear to bind CD40 directly (Pullen et al., 1998). It is probable that TRAF1 and TRAF2 act as a heterodimeric complex to activate the canonical NFκB pathway (Xie et al., 2006).

Finally, CD40 stimulation results in increased expression of anti-apoptotic factors such as BCL-XL, A20, BFL-1, survivin and cFLIP (Davies et al., 2004) (Figure 1.5).

In murine B cells, activation of the AKT (but not the MAPK or the NFκB) signalling pathway has been shown to be dependent upon CD40 and lipid raft association, which is independent of any signalling events (Nadiri et al., 2011).

A gene expression profiling study showed that stimulating mouse B cells with CD40 resulted in gene expression changes in multiple signalling pathways (Dadgostar et al., 2002). They study showed that post CD40 stimulation the most up-regulated primary response genes are involved in the NF-κB pathway whereas most down-

regulated genes are involved in the p38 pathway. Genes involved in PI3K pathway are also upregulated. They suggested that the ERK pathway plays little role in the early changes in gene expression mediated by CD40.

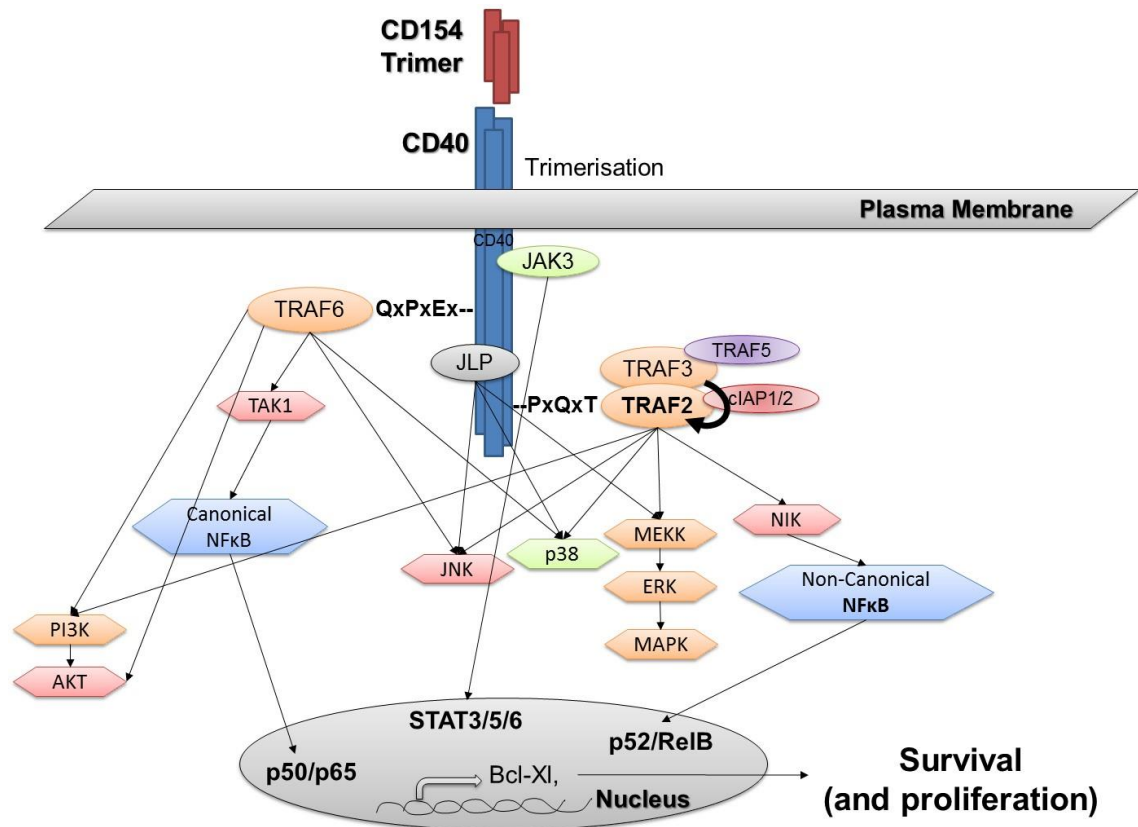


Figure 1.5: CD40 signalling.

CD154-CD40 interaction is generally thought to occur in a trimerized fashion. CD40 cross-linking probably multimerizes TRAF proteins which may act as direct transcriptional regulators. CD154 engagement of CD40 results in the recruitment of adapter proteins known as TNFR-associated factors (TRAFs) to the cytoplasmic domain of the CD40 receptor. TRAF1, 2, 3, 5 and 6 can all be recruited to the intracellular tail of CD40. JAK3 has also been shown to bind directly to CD40 in some cells, resulting in STAT5 phosphorylation. TRAF6 binds to the proximal amino acid sequence QxPxEx, and activates TAK1 which promotes the NFκB canonical pathway, PI3K, p38 and JNK signalling pathways. TRAF2 and TRAF3 bind to the same distal region, demarcated by the amino acid sequence: PxQxT. CD40-induced activation of TRAF2 induces cIAP1 proteins to ubiquitylate TRAF3, promoting its degradation and releasing MEKK1 and stable expression of autocatalytic NIK for downstream signalling, leading to activation of the non-canonical NFκB pathway. TRAF2 and TRAF3 also activate p38 and JNK and AKT signalling pathways. TRAF5 associations with CD40 are indirect via TRAF3. In the absence of TRAF2, TRAF1 can bind the distal TRAF2-binding site, but in normal circumstances it does not appear to bind CD40 directly. CD40 internalization is a JLP-mediated process of vesicle transportation that depends on Rab5 and dynein. Finally, CD40 stimulation results in increased expression of anti-apoptotic factors such as BCL-XL, A20, BFL1, survivin and cFLIP. Figure based on reviews (van Kooten and Banchereau, 2000, Elgueta et al., 2009).

1.1.4.2.1.3 CD40 in CLL

In vitro studies where CLL cells are stimulated with either soluble CD40L (sCD154) or membrane-bound CD40 (mCD154) showed slightly different results. sCD154 and

mCD154 both promoted CLL-cell survival and prevented drug-induced apoptosis, but mCD154 also promoted CLL-cell proliferation. CLL cells express some low levels of CD154 in one third of cases, and thus can create an autocrine or paracrine pathway to sustain cell survival and prevent apoptosis as well as driving proliferation (Schattner, 2000).

CLL cells do show a differential response to sCD154 although the exact reason is unclear. sCD154 leads to the upregulation of CD95 and CD80 and chemokines CCL22 and CCL17 in some cases, but not in others. In the cases where it did produce chemokines and up-regulate surface markers, signalling was occurring via the NFκB pathway resulting in upregulation of BCL-2 and MCL1. These sCD154-responsive cases had lower mean lymphocyte counts in peripheral blood and longer time to treatment progression (Scielzo et al., 2011).

CD40 stimulation in CLL cells activates NFκB (Schattner, 2000, Furman et al., 2000), which results in increased expression of anti-apoptotic factors such as BCL-2, BCL-XL and BFL1, c-IAP1 and c-IAP2, leading to inhibition of apoptosis. CD95 is also upregulated upon CD40 stimulation, but CLL cells were not sensitive to CD95L-induced apoptosis (Kater et al., 2004). CD40 stimulation of CLL cells results in resistance to chemotherapeutic agents, such as fludarabine (Romano et al., 1998). The induction of BCL-XL and BCL2-A1 upon CD40 stimulation resulted in resistance to apoptosis induced by BCL-2 inhibitor ABT-737 (Vogler et al., 2009a).

CD40 + IL-4R stimulation of CLL cells, appears to upregulate CD38 and ZAP-70 protein expression, which both are known to have prognostic significance (Willimott et al., 2007) and increase TACI and BCMA expression, receptors for BAFF and APRIL respectively (Ferrer et al., 2014).

CD40 stimulation alone can result in CLL-cell proliferation in a small number of cases. Thus it has been shown that after five days co-culture with CD154-expressing 3T3 fibroblasts, proliferation was induced in 30% UM-CLL cells (Tromp et al., 2010).

In terms of CD40 signalling in CLL cells, there is incomplete information in the literature. It has been shown that TRAF1 was expressed at a higher level in CLL

cells than normal B cells, but the other TRAFs were equally expressed (Zapata et al., 2000).

1.1.4.2.1.4 CD40 stimulation-induced gene expression in CLL.

The gene expression profile of CD40-stimulated CLL cells was observed to be similar to that in CLL cells stimulated by activated T cells. The induction of apoptosis-related genes, including BCL-XL, BID, CD95, c-IAP1, BCL2-A1, p21 and SERPINB9 was also observed in CLL cells stimulated by both CD154 and activated T cells (Pascutti et al., 2013).

Normal B cells proliferate faster than CLL cells upon CD40 stimulation. To understand this difference, gene expression array studies were performed to examine the gene expression of CLL cells versus healthy B cells after CD40 activation. Many genes involved in cell cycle regulation were up-regulated in both CLL cells and healthy B cells. However, *DUSP4* and *ZNF151* genes, both of which have growth inhibitory functions, were upregulated in CLL cells, but not healthy B cells (Gricks et al., 2004).

1.1.4.2.1.5 Targeting CD154-CD40 interaction in CLL

A study attempting to inhibit the CD154-CD40 interaction using a humanised anti-CD40 antagonist antibody, HCD122, showed that the antibody not only inhibited CD40-induced proliferation, survival and secretion of cytokines but also promoted antibody-dependent cellular cytotoxicity (ADCC)-mediated killing of CLL cells *in vitro* (Luqman et al., 2008). However, clinical efficacy of this antibody remains to be established.

1.1.4.2.2 B cell receptor (BCR) signalling

There are many recent reviews comprehensively covering B cell receptor (BCR) signalling in CLL (Robak and Robak, 2013, Hill et al., 2013, Rickert, 2013, Fowler and Davis, 2013, Woyach et al., 2012, Wiestner, 2012). Other reviews include those by researchers who have dedicated almost their entire career to studying the role of BCR signalling in the biology of CLL (Packham and Stevenson, 2010, Stevenson et al., 2011, Packham et al., 2014). Here I provide a brief summary on BCR signalling in CLL.

1.1.4.2.2.1 Evidence for the importance of BCR signalling in CLL

BCR signalling is thought to be an important pathway in driving the expansion of CLL malignant clones. A role for antigenic stimulation in CLL is inferred from the observation that CLL cells use a restricted repertoire of IGHV genes (Agathangelidis et al., 2012, Fais et al., 1998, Messmer et al., 2004a, Messmer et al., 2004b, Tobin et al., 2004). Whilst this evidence alone does not prove BCR signalling is involved in CLL, the fact that BCR stimulation enhances survival and to some degree proliferation in CLL and that the degree of BCR activation predicts clinical outcome suggests BCR signalling is important in CLL (Le Roy et al., 2012).

1.1.4.2.2.2 BCR signalling in normal B cells

The BCR complex is made up of the heavy and light chain immunoglobulin non-covalently bound to CD79A and CD79B. Upon antigen binding, the BCR oligomerizes and the immunoreceptor tyrosine-based activation motifs (ITAMs) of CD79A and CD79B are phosphorylated by LYN (Figure 1.6). LYN is the main SRC kinase in B cells, though in CLL cells ZAP70 also acts as a SRC family kinase and increases BCR signalling (Chen et al., 2002). Phosphorylated ITAMs promote Spleen tyrosine kinase (SYK) binding to those motifs. LYN also phosphorylates SYK at a regulatory motif (Figure 1.6).

BLNK/SLP-65 is recruited to a non-ITAM portion of CD79A, where it is phosphorylated by SYK and acts as a scaffold protein. LYN also phosphorylates YXXM motifs in CD19, which facilitates recruitment of PI3K, via its regulatory p85 subunit. PI3K then generates PIP₃ which recruits AKT and BTK to the plasma membrane via their PH domains. BTK activation is initiated by plasma membrane association and phosphorylation of Y551 by SYK. This phosphorylation results in the catalytic activation of BTK and leads to its autophosphorylation at position Y223 in the SH3 domain (Figure 1.6).

Eventually a complex of signalosome forms consisting of LYN, SYK, PI3K, BTK, PLC- γ 2, and BLNK/SLP-65 (Figure 1.6). VAV is also recruited into this signalosome complex and plays a role in RAC activation. BTK phosphorylates PLC γ 2 at Y753 and Y759, which is essential for its lipase action. PLC γ 2 cleaves PIP₂ to generate IP₃ and DAG. IP₃ increases intracellular calcium levels, thereby modulating calmodulin which activates the NFAT (nuclear factor of activated T

cells) transcription factors. DAG activates PKC β , which induces NF κ B signalling via the CBM complex, composed of CARMA1/CARD11, BCL-10 and MALT1. DAG and PKC β together activate RASGRP1, which activates the RAS-ERK pathway. BTK can also recruit PI3K, thereby creating more PIP₃ and generating a positive feedback loop.

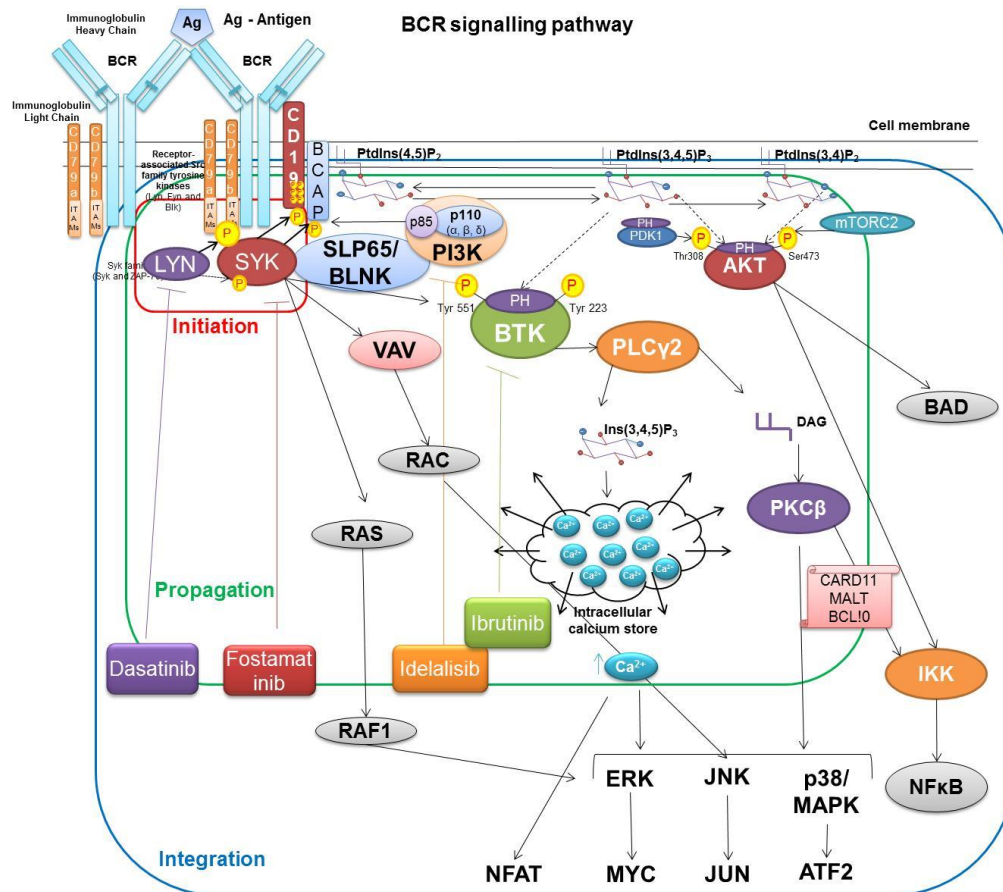


Figure 1.6: B-cell receptor signalling and targeted inhibition

B cell antigen receptor signal transduction cascade. Signal transduction initiation occurs at the cell membrane following ligand-induced aggregation of the membrane immunoglobulin (mIg) and associated signal transducing elements CD79A and CD79B and SRC kinases LYN and SYK. Propagation of signals then occurs by protein phosphorylation, modification, and interaction. Integration of these signals culminates in the regulation of transcription factor activation and gene expression. The diagram shows the major signalling pathways activated after BCR activation. Chemical inhibition of LYN, SYK, BTK, and PI3K by Dasatinib, Fostamatinib, Ibrutinib or Idelalisib respectively, blocks BCR signalling. Abbreviation definitions are included in the accompanying text. Figure based on a review (Dal Porto et al., 2004).

1.1.4.2.2.3 BCR signalling in CLL cells

There are several key differences in BCR signalling between normal B and CLL cells. Firstly, CLL has very low expression of surface IgM and CD79B. The response to BCR stimulation in CLL cells is variable. There is increased expressed of LYN, SYK and ectopic expression of ZAP70 in CLL. The levels of ZAP70 can be used as

a prognostic indicator for poor prognosis (Chen et al., 2002, Contri et al., 2005, Buchner et al., 2009). Compared with normal B cells, CLL cells also express increased levels of phosphorylated SYK, NFκB, and ERK in the absence of stimulation by extrinsic antigens, suggesting that antigen-independent BCR signalling may be occurring in CLL cells (Hill et al., 2013). UM-CLL cells had prolonged SYK activation as compared to M-CLL cells.

The expression level of PI3K p110δ is similar between normal B cells and CLL cells, but the kinase activity is higher in CLL (Herman et al., 2010). The extent of calcium flux in CLL cells is variable from case to case and generally low. Generally, ZAP70+ cells respond to IgM stimulation better than ZAP70- cells *in vitro* (Woyach et al., 2012).

M-CLL is mainly, but not exclusively driven towards anergy *in vivo*. In contrast, UM-CLL shows less evidenced for anergy *in vivo* retaining more responsiveness to surface IgM mediated signalling (Packham et al., 2014). In *in vitro* studies of BCR activation by crosslinking the receptor with anti-IgM antibodies, M-CLL cells are often considered ‘anergized’ as they don’t respond to such activation as well as UM-CLL cells (Guarini et al., 2008). Anergy is defined by a failure to respond to BCR-mediate stimuli, which is a component of normal B cell behaviour. It is a state of cellular lethargy resulting from binding of antigen by B cells in the absence of significant CD4+ T-cell help (Cambier et al., 2007).

Less is known about the BCR signalling in anergic CLL cells. Chronic BCR signalling in anergic B cells is associated with mono-phosphorylation of CD79A/B rather than dual-phosphorylation. It also results in raised basal ERK phosphorylation, raised basal intracellular calcium levels and activation of NFAT without activation of other kinases (such as AKT or NFκB). SHIP1 seems to be constitutively phosphorylated, and this may play a role in inhibition of other receptors. Anergy is also associated with suppression of remote receptors such as CXCR4 (which binds and responds to CXCL12) and TLR9 (which responds to CpG-ODN). There is also increased basal pro-apoptotic protein BIM expression in anergic CLL cells. (This topic is well reviewed by Packham and colleagues: (Packham et al., 2014)).

However, *in vivo* data from another group suggested that both types of CLL cells can undergo antigen-dependent BCR activation (Herishanu et al., 2011). Activation of

the BCR on CLL cells induces a characteristic gene expression signature that consists of sixty-one genes irrespective of *IGVH* status, although these genes are up-regulated to higher levels in UM-CLL than M-CLL cells (Herishanu et al., 2011).

In an attempt to identify specific ligands to BCRs of both M-CLL and UM-CLL cells, Seiler and colleagues used a phage-displaying peptide library screening method to show that the surface membrane Ig of M-CLL cells tend to bind peptides with well defined amino acid motifs with high specificity whereas the surface membrane Ig of UM-CLL cells recognise multiple different epitopes with low affinity (they can be considered promiscuous) (Seiler et al., 2009). Their data suggest that the *in-vivo* structurally diverse epitopes could bind surface membrane immunoglobulins of different CLL clones and trigger varied BCR responses in these cells, thus resulting in distinct outcome on survival and growth.

1.1.4.2.2.4 Targeting BCR signalling

BCR signalling has become an important therapeutic axis in CLL. Drugs that target the BCR signalling components LYN, SYK, BTK, and PI3K include dasatinib, fostamatinib, ibrutinib and idelalisib respectively, and are already in clinical development (see [section 1.1.5.3](#)).

1.1.4.2.3 Bone marrow stromal cells

Bone marrow stromal cells (BMSCs) were the first stromal cells shown to support CLL cells *in vitro* (Lagneaux et al., 1998). CLL cells have a high affinity for BMSCs and CXCR4-CXCL12 signalling is critical for this interaction. CLL cells adhere to, and a small fraction often spontaneously migrate underneath, the BMSCs (Burger et al., 1999). Very Late Antigen-4 (VLA-4) integrins, also known as CD49d/CD29, cooperate with CXCR4 in CLL-cell adhesion to BMSCs (Burger, 2011). BMSCs protect CLL cells from spontaneous and drug-induced apoptosis (Kurtova et al., 2009, Nwabo Kamdje et al., 2012) and hence are considered to be a tumour ‘sanctuary’ and contribute to drug resistance (Meads et al., 2008).

1.1.4.2.4 Nurse-like cells.

Monocytes from CLL PBMCs differentiate *in vitro* into large, round, adherent cells that attract CLL cells. These “nurse like cells” (NLCs) then protect CLL cells from undergoing spontaneous or drug-induced apoptosis. The interactions between NLCs

and CLL cells involve both direct contact and the release of soluble factors (Burger, 2011). A whole range of chemokines, cytokines and TNF family ligands are involved in the NLC-CLL interaction. NLCs express CXCL12, CXCL13, CD31, BAFF and APRIL which interact with their cognate receptors CXCR4, CXCR5, CD38, BAFFR and BCMA and TACI on CLL cells (Burger et al., 2000, Nishio et al., 2005). CLL cells produce T-cell chemokines CCL3 and CCL4 after co-culture with NLCs (Burger et al., 2009). It is thought that CCL3/CCL4 protein secretion by CLL cells upon NLCs occurs via BCR activation, since inhibiting SYK inhibited their secretion. BCR stimulation alone was also shown to increase CCL3/CCL4 secretion. This shows how through chemokines, CLL cells can recruit accessory cells, such as T cells to create a supportive microenvironment (Burger et al., 2009).

1.1.4.2.5 Endothelial cells

CLL cells also interact with vasculature. Immunohistochemical staining has demonstrated the presence of abundant blood vessels in CLL lymph tissues. CLL cells were shown to produce vascular endothelial growth factor (VEGF₁₂₁ and VEGF₁₆₅) and VEGF was able to attract endothelial cells, which may be relevant for the tissue phase of the disease (Chen et al., 2000).

1.1.4.2.6 Cross-talk in the tissue microenvironment

There is debate about which interactions in the tissue microenvironments are the most important and thus which *in-vitro* models best mimic the CLL microenvironment (Burger and Gandhi, 2009). Whilst there is no single model that fully recapitulates the *in-vivo* microenvironment, there is strong evidence indicating a role of interaction of T cells with CLL cells via CD154-CD40 *in-vivo*.

In addition, there is cross-talk between different pathways. For example, CD40 signalling has been shown to increase the levels of CD79b, a co-adaptor of BCR, thereby increasing signalling activity of BCR pathway (Minuzzo et al., 2005).

The CD40 and BCR signalling pathways share multiple components, such as protein tyrosine kinases SYK, LYN, PI3K and AKT, PLC γ (Faris et al., 1994, Ren et al., 1994, Ying et al., 2011, Bishop et al., 2007). There are two studies suggesting that the BCR and CD40 signalling pathways can converge (Ying et al., 2011, Os et al., 2013).

1.1.4.3 CLL-cell apoptosis and proliferation

It is well known that when CLL cells isolated from the peripheral blood of a patient are cultured *in vitro*, a substantial proportion of them spontaneously die by apoptosis. Collins initially observed that about 20% of cultured CLL cells lost viability over a 30 hour incubation period and that addition of T cells or autologous serum did not affect viability (Collins, 1989). This suggests that CLL cells retain the biochemical machinery needed to execute apoptosis and that their prolonged survival *in vivo* requires microenvironmental factors present at sites of tissue involvement including bone marrow and lymph nodes. UM-CLL cells are more prone to spontaneous apoptosis than their M-CLL counterparts under culture conditions *in vitro*, suggesting that survival of UM-CLL cells is more dependent on microenvironment *in vivo* (Coscia et al., 2011).

1.1.4.3.1 Regulation of apoptosis of CLL cells by BCL-2 family of proteins

Despite the fact that CLL cells apoptose rapidly *in vitro*, CLL cells clearly accumulate *in vivo* and are often resistant to apoptosis after exposure to chemotherapeutic agents. This suggests that their enhanced survival and resistance to apoptosis is dependent on the protective microenvironment *in vivo*. The molecular mechanisms that regulate apoptosis in CLL are complex, but the importance of the BCL-2 family of proteins is clear.

Within the BCL-2 family of proteins, there are five anti-apoptotic proteins BCL-2, BCL-XL, MCL1, BFL1/A1 (also known as BCL2-A1) and BCL-W, and two major pro-apoptotic effector proteins BAX and BAK. They all contain multiple BCL-2 homology (BH) domains. In addition, there are several pro-apoptotic BH3-only proteins within the BCL-2 family that act either as activators, such as BID, BIM, PUMA, or sensitizers such as BAD, BMF and NOXA (Adams and Cory, 2007, Youle and Strasser, 2008, Chipuk et al., 2010, Czabotar et al., 2014) (Figure 1.7).

Exactly how the family members exert their effects in apoptosis is still being intensely investigated. Nevertheless, they appear to control the permeabilisation of the outer membrane of mitochondria, which results in release of cytochrome c, activation of caspase-9 and triggering of the caspase cascade, leading to the morphological and biochemical changes associated with apoptosis. There are

currently two models in which the BH3-only proteins activate apoptosis. In the direct model, the BH3-only pro-apoptotic activators directly interact with BAX or BAK. Activated BAX or BAK can then oligomerise to form pores on mitochondrial outer membrane resulting in its permeabilisation. In the indirect model, the BH3-only proteins cannot bind and activate BAX or BAK directly; instead they sequester anti-apoptotic BCL-2 family proteins, thereby allowing the release of pro-apoptotic effector molecules BAX or BAK (Figure 1.7).

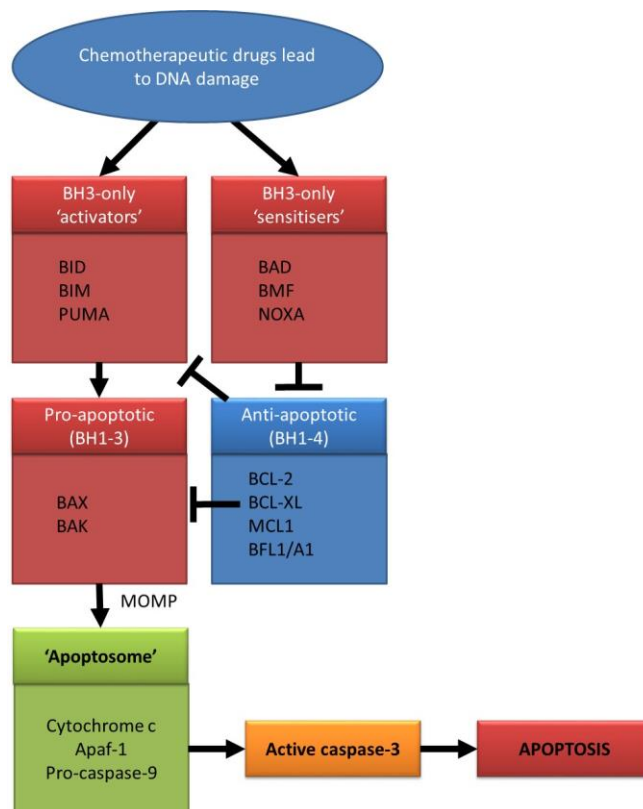


Figure 1.7: Model of the regulation of apoptosis by Bcl-2 family proteins.

The anti-apoptotic proteins BCL-2, BCL-XL, BCL-W, MCL1 and BFL-1/A1 all have four BH domains and interact with other BCL-2 family proteins to prevent mitochondrial outer membrane permeabilisation (MOMP). The pro-apoptotic proteins are divided into two subgroups: the first includes BAX and BAK which have three BH3 domains (BH1–3) whereas the second includes BIM, BID, BAD and NOXA and PUMA which only share sequence homology with the BH3 domain and hence are known as BH3-only proteins. The current thinking is that BH3-only proteins are activated in response to numerous cells death stimuli and these cause the activation of BAX or BAK. Only following activation can BAX and BAK homo-oligomerise and initiate MOMP leading to the release of cytochrome c and the downstream activation of caspases. There are currently two models in which the BH3-only proteins activate apoptosis. In the direct model, the BH3-only pro-apoptotic activators directly interact with BAX or BAK. Activated BAX or BAK can then oligomerise to form pores on mitochondrial outer membrane resulting in its permeabilisation. In the indirect model, the BH3-only proteins cannot bind and activate BAX or BAK directly; instead they sequester anti-apoptotic BCL-2 family proteins, thereby allowing the release of pro-apoptotic effector molecules BAX or BAK. Based on text and figure 2 from review (Buggins and Pepper, 2010).

In CLL, the anti-apoptotic BCL-2 protein is overexpressed compared to normal controls (Pepper et al., 1997, Pepper et al., 1998, Kitada et al., 1998). Further, BCL-2

was particularly pronounced in those patients found to be clinically unresponsive to chemotherapy. Overexpression of BCL-2 is an independent prognostic factor for poor outcome of the disease (Robertson et al., 1996, Faderl et al., 2002). MCL1, another anti-apoptotic BCL-2 protein, is also highly expressed (Pedersen et al., 2002, Longo et al., 2008, Bannerji et al., 2003). These anti-apoptotic BCL-2 family proteins (BCL-2, BCL-XL, MCL1 and BFL1/A1) are also highly expressed in CLL cells directly obtained from lymph node samples of patients (Smit et al., 2007, Hallaert et al., 2007) and in CLL cells co-cultured with bone marrow stromal cells, nurse-like cells and CD40 ligand-expressing fibroblasts *in vitro*, leading to resistance to apoptosis induced by chemotherapeutic agents (Kitada et al., 1999, Burger et al., 2005, Vogler et al., 2009a, Zhuang et al., 2014).

Whilst co-culture of CLL cells with CD40L-expressing fibroblasts protects from apoptosis induced by chemotherapeutic agents, PI3 kinase inhibitor, idelalisib, was able to abrogate the protective effect provided by CD40L, by reducing AKT phosphorylation and MCL1 expression (Herman et al., 2010), whilst the BTK inhibitor, ibrutinib also abrogated the protection induced by CD40L (Herman et al., 2011a). Immunotherapy, such as type II CD20 mAb GA101, was shown *in vitro* to sensitise CLL cells to various cytotoxic drug induced cell death upon CD40 stimulation (Jak et al., 2011). In lymphoma cells, rituximab (a type I CD20 mAb) was shown to inhibit the p38 MAPK and NFκB pathways and downregulate the expression of anti-apoptotic BCL-2 family proteins, which may explain the mechanism by which GA101 sensitises chemotherapeutic agents in CLL cells (Alas and Bonavida, 2001, Vega et al., 2004, Jazirehi et al., 2005).

Drug resistance in CLL is thought to be driven to a large extent by either p53 dysfunction or the imbalance in expression of apoptosis-regulating proteins (Buggins and Pepper, 2010, Packham and Stevenson, 2005). High levels of BCL-2 and low levels of BAX results in drug resistance (Pepper et al., 1999). The pro-apoptotic BH3-only proteins PUMA, NOXA and BIM are required for apoptosis induced by some cytotoxic drugs currently used for CLL treatment and enforced expression of these proteins is able to restore sensitivity of CLL cells to chemotherapeutic agents (Zhang et al., 2013). The pro-apoptotic BCL-2 family of proteins BAX, PUMA, NOXA and BID are also transcriptional targets of p53, therefore the levels of these proteins is low in CLL cells with dysfunctional p53 and these cells are usually not

responsive to induction of apoptosis by cytotoxic agents that is dependent on p53 (Zhang et al., 2013, Buggins and Pepper, 2010).

BH3 mimetic molecules, which mimic the action of BH3-only proteins by binding to the hydrophobic groove of the anti-apoptotic BCL-2 family of proteins, such as BCL-2, are now being evaluated for clinical development in CLL (see [section 1.1.5.5](#)).

1.1.4.3.2 CLL - a disease of accumulation

CLL was classically thought to be a disease of accumulation of malignant B cells, due to defects in apoptosis (Dameshek, 1967). However, this dogma began to be challenged amid the emerging evidence that CLL is a proliferative disease. Firstly, during the clinical course of the disease some CLL clones acquired genetic mutations and chromosomal abnormalities, leading to clonal expansion (Landau et al., 2013). This suggests that proliferation is actively taking place. Secondly, there was a robust correlation between the level of mutations and telomere lengths, which are used as a surrogate variable for cell turnover (Damle et al., 2004). This again suggests that proliferation of CLL cells occurs. The definitive evidence that CLL is a proliferative disease came from clinical observations using a safe, non-radioactive *in vivo* labelling method that permitted the determination of CLL-cell birth rates, as described below.

1.1.4.3.3 CLL - a proliferative disease.

In a seminal study, a stable isotopic labelling method was used where patients drank deuterated water ($^2\text{H}_2\text{O}$) for 84 days and the deuterated hydrogen incorporated into the deoxyribose moiety of DNA was used to monitor CLL cell kinetics *in vivo* (Messmer et al., 2005). It was found that CLL cell birth rates of each patient varied between 0.1-1.0% per day and that those patients with birth rate greater than 0.35% per day were more likely to have or develop progressive disease than those with lower birth rates.

This study and others published afterwards showed that variation between kinetics in different cases of CLL was associated with prognostic indicators – e.g. high CD38 expression and unmutated *IGVH* had higher proliferation rates (two-fold higher in

the case of high CD38, and two-to-three fold higher in the case of unmutated *IGVH*) (Calissano et al., 2009, van Gent et al., 2008).

It is interesting to note that CLL cells proliferate and die at a slower rate than normal B cells *in vivo*. Average turnover rates of 0.08–0.35% of the clone per day in CLL cells was observed, which was two-fold lower than average turnover rates in B-cells from healthy individuals (0.34–0.89%). Similarly, labelled CLL cells in blood disappeared at a rate of 0.00–0.39% per day, which was 10-fold lower than that in labelled B cells from healthy individuals (1.57–4.24% per day) (van Gent et al., 2008).

Additional evidence was also obtained from an immunohistochemistry study using tissue microarray technology examining paraffin-embedded specimens of lymph nodes from CLL patients (Obermann et al., 2007). It showed that 15.66% of cells have entered the G1-phase of cell cycle, suggesting a proliferative potential in a substantial proportion of the CLL-cell population.

1.1.4.3.4 CLL cell cycle

In the classical model of the cell cycle, cells proceed from G1 (growth phase, when the cells commit to cycling through the cell cycle), to S phase (DNA replication occurs), G2 phase (further growth, when cells can repair errors that occur during DNA synthesis) and finally M phase or mitosis (when breakdown of the nuclear envelope occurs and cytokinesis takes place) (Hochegger, 2008). The transition between these phases is controlled by cyclins and cyclin dependent kinases (CDKs). Cyclin dependent kinases (CDKs) trigger the transitions by phosphorylating distinct sets of substrates. Cyclin D (1-3) and CDK4 or CDK6 regulate events in early G1 phase, cyclin E-CDK2 triggers S phase, cyclin A-CDK2 and cyclin A-CDK1 regulate the completion of S phase, and CDK1-cyclin B is responsible for mitosis.

Additional control of the entry into S phase is regulated by the CDK inhibitors (CDKis). There are two families of CDKis based on their structures and CDK targets. The CIP/KIP family of CDKis consists of p21, p27 and p57 and the INK4 family of proteins (inhibitors of CDK4) which are named for their ability to specifically inhibit the catalytic subunits of CDK4 and CDK6 (but not other CDKs or D-type cyclins). This family includes p16, p15, p18 and p19. INK4 CDKis are

composed of multiple ankyrin repeats. The CIP/KIP CDKs: p21, p27 and p57 have broader activity and inhibit cyclin D-, E-, and A-dependent kinases. CIP/KIP CDKs contain characteristic motifs within their amino-terminal moieties that enable them to bind both to cyclin and CDK subunits (Sherr and Roberts, 1999).

CLL cells isolated from the peripheral blood are largely in G0 phase of cell cycle, most probably due to high levels of p27 (Vrhovac et al., 1998). p27 binds to CDK2 and cyclin E complexes to prevent cell-cycle progression from G1 to S phase. p27 is 2-fold higher in 80% of CLL compared to normal B cells (Korz et al., 2002). p27 levels decrease rapidly in B cells when stimulated with proliferative cytokines such as IL-4 (Blanchard et al., 1997) and p27 is degraded in a ubiquitin-dependent fashion, since proteasome inhibitors block this degradation (Bogner et al., 2003). The expression of p27 can be negatively modulated by the miR-221/222 cluster, as stimulating CLL cells with CpG-ODN or CD154 led to increased expression of miR-221/222 and downregulation of p27 (Frenquelli et al., 2010).

Counterintuitively, p27 mRNA correlated with Rai staging ($R=0.3$, $p=0.066$) (Halina et al., 2010). High expression of p27 has been found to predict rapid progression of the disease (Wolowiec et al., 2009). High p27 levels correlate with shorter lymphocyte and total tumour mass doubling time and lead to shorter survival (but also increased viability in culture) (Vrhovac et al., 1998). Exactly why high p27 mRNA and protein levels associate with poor prognosis is unknown.

Cyclin D1 is not detectable in resting or activated normal B or CLL cells, but both Cyclin D2 and D3 are present in CLL cells (Decker et al., 2002). Early cell cycle progression differs between CLL cells and normal B cells. Cyclin D2 mRNA is overexpressed five-to-ten-fold in CLL, compared to normal B cells (Delmer et al., 1995). Cyclin D2 has been found to be overexpressed in lymph nodes of patients with CLL. Cyclin D2 positive cells were also Ki67 positive, i.e. they were in the S-phase of cell cycle. Cyclin D2 overexpression was in part due to an upregulation of NF κ B, but also c-Myc (Igawa et al., 2011). The CCND2 gene is located on chromosome 12p13 and many CLL patients have trisomy 12 (Igawa et al., 2011). The D cyclins bind to CDK4 and CDK6, leading to the phosphorylation of retinoblastoma protein, RB and to G1/S phase transition. Both cyclin D2 and D3 associate with CDK4 in CLL cells. CDK4 has been shown to be overexpressed in

CLL cells compared to normal B cells and this higher level of expression has been associated with lower survival rates in CLL (Winkler et al., 2010).

1.1.4.3.5 CLL cells proliferate primarily in lymph nodes not bone marrow

The *in-vivo* dynamics study of proliferation suggested that the lymph node rather than the bone marrow was the major tissue site where CLL proliferation takes place (van Gent et al., 2008). Subsequent gene expressing profiling of CLL samples from peripheral blood, bone marrow and lymph node strengthened this suggestion (Herishanu et al., 2011). CLL cells in the lymph nodes had up-regulated expression of genes involved in BCR signalling and NFκB and NFAT activation. These cells were also shown to be undergoing more proliferation as measured by E2F, c-myc and Ki67. NFκB target genes involve cell cycle regulation (*CCDN2*, cyclin D2), inhibition of apoptosis (*BCL2A1*), signal transduction (*JUNB*, *DUSP2*) and chemotaxis (*CCL3*, *CCL4* and *RGS1*). Herishanu and colleagues thus developed a proliferation score by averaging the expression of the most highly enriched E2F target genes and showed that the proliferation score was much higher in lymph node CLL cells than bone marrow CLL cells (Herishanu et al., 2011). This was further confirmed by an independent study showing that the proliferation score was significantly higher in lymph node-derived CLL cells than bone marrow-derived cells (Pascutti et al., 2013).

1.1.4.3.6 *In-vitro* models of CLL proliferation

In order to study the molecular mechanisms regulating CLL-cell proliferation, there have been many efforts to establish *in vitro* models. CpG-ODN, which mimics CpG motifs present in unmethylated bacterial DNA, can engage toll-like receptor 9 (TLR9) on CLL cells. CpG-ODN is known to activate NFκB, PI3K/AKT, JNK, ERK1/2 and p38MAPK pathways in CLL cells (Decker et al., 2000b). It has been shown that the addition of IL-2 to CpG-ODN can assist CLL cell proliferation (Jahrsdorfer et al., 2005, Decker et al., 2000a).

Another stimulus able to induce CLL-cell proliferation is CD40 ligand (also known as CD154), either in the soluble form (sCD40L), or the immobilised form that is expressed on the cell surface of transfected cells such as HeLa cells (HeLa-CD154) or mouse fibroblast (3T3-CD154). CD40 stimulation alone is not potent in

inducing CLL-cell proliferation (Tromp et al., 2010). Thus additional cytokines are usually required such as IL-4 (Buske et al., 1997, Frenquelli et al., 2010, Palacios et al., 2014) or IL-4 + IL-21 (Ahearne et al., 2013). Pascutti and colleagues suggested that CD154 + IL-21 might represent the better model of CLL-cell proliferation since IL-21 is detected in the lymph node and it, together with CD40 stimulation, induced more proliferation than CD154 + IL-4, CD154 + IL-15, CD154 + IgM or CpG+IL-2 (Pascutti et al., 2013).

Plander and colleagues showed that CLL cells from progressive clinical cases were induced to proliferate by a combination of soluble CD154/IL-2/IL-10 in co-culture with stromal cells (Plander et al., 2009). Fecteau and colleagues, whilst investigating the anti-proliferative effect of lenolidomide, used membrane-bound CD154 + IL-2 + IL-10 to induce proliferation of CLL cells (Fecteau et al., 2014).

1.1.5 Current clinical treatment

Despite the significant advancement in the development of novel therapies in the treatment of CLL, the disease still remains incurable without allogeneic haematopoietic stem cell transplantation, which has high risk of developing graft versus host disease. Despite CLL being incurable, there are many effective ways of managing the disease. Current general guidelines for treatment are summarised below.

When a patient is classified as Binet stage A-B, or Rai 0-II, 'watchful waiting' is generally considered as the first option with no treatment given. Once the disease has progressed to Binet stage C, or Rai stage III-IV, then treatment is advisable.

If the patient is young and healthy and does not have a chromosomal deletion in 17p, the current standard treatment is an immunochemotherapy consisting of a combination of fludarabine (F), cyclophosphamide (C) and rituximab (R) (Hallek, 2013, Jain and O'Brien, 2015). If the patient cannot tolerate the toxicity of the FC then chlorambucil in combination with an anti-CD20-monoclonal antibody such as rituximab may be prescribed (Hallek, 2013, Jain and O'Brien, 2015).

If the patient has a chromosomal deletion in 17p or a p53 mutation and is fit, allogeneic haematopoietic stem cell transplantation is recommended. If the patient is unfit, the options include use of an antibody-based therapy such as alemtuzumab,

rituximab or ofatumumab (Hallek, 2013). Idelalisib or Ibrutinib may be used as front line treatments in this high risk group (Hallek, 2013, Jain and O'Brien, 2015).

It is worth emphasising that most patients will relapse after initial treatment. The general guidance is to repeat the first line of therapy. Otherwise, depending on the fitness of patients, there are a whole host of treatment possibilities (Hallek, 2013).

Below I shall outline the main treatments available and their modes of action. The main therapies and their molecular modes of action are summarised in Table 1.6.

1.1.5.1 Chemotherapies

1.1.5.1.1 Chlorambucil

Chlorambucil (CLB) is an alkylating agent, which was used as frontline monotherapy for CLL for many decades before being superseded by fludarabine-cyclophosphamide (FC). Alkylation of DNA causes developing DNA interstrand cross-links, thus preventing replication and inducing apoptosis, which has been considered the main mechanism responsible for the cytotoxicity of chlorambucil (Panasci et al., 2001). Even today, this drug is still useful, particularly in 'unfit' patients as it is less toxic than FC and inexpensive. However, CLB, which is administered orally, is now recommended to be used in combination with anti-CD20 antibodies, e.g. rituximab or ofatumumab, for treatment of older patients unfit for standard treatment (Goede et al., 2015a, Hillmen et al., 2014, Hillmen et al., 2015).

Table 1.6: Current and up-coming therapeutics for CLL.

Class of drug	Sub class of drug	Name of drug	Abbreviated drug name	Mode of Action
Chemotherapies		Chlorambucil	CLB	<ul style="list-style-type: none"> •Alkylating of DNA, causing DNA intrastrand and interstrand cross-links, resulting in inhibition of DNA replication, repair and transcription.
		Fludarabine	F	<ul style="list-style-type: none"> •Fluorinated analogue of adenine – results in inhibition of DNA and/or RNA synthesis •Inhibition of ribonucleotide reductase and DNA polymerase, thereby inhibiting DNA synthesis and repair. •Activates the mitochondrial apoptosis pathway.
		Cyclophosphamide	C	<ul style="list-style-type: none"> •Alkylating of DNA.
		Bendamustine	B	<ul style="list-style-type: none"> •Alkylating of DNA. •Unique mechanisms.
Immunotherapy (Antibody therapy)	Anti-CD20 antibodies	Type I - Rituximab and Ofatumumab	R and O	<ul style="list-style-type: none"> •Antibody Dependent Cellular Cytotoxicity (ADCC) which utilises immune cells bearing Fc receptors. •Antibody Dependent Cellular Phagocytosis (ADCP). •Clusters CD20 into lipid microdomains and potentially activates complement dependent cytotoxicity (CDC). •Does not induce apoptosis directly.
	Anti-CD20 antibodies	Type II – Obinutuzumab (GA-101)		<ul style="list-style-type: none"> •Antibody Dependent Cellular Cytotoxicity (ADCC). •Antibody Dependent Cellular Phagocytosis (ADCP). •Does not activate CDC. •Strong inducer of apoptosis.
BCR signalling inhibitors	BTK inhibitors	Ibrutinib	PCI-32765	<ul style="list-style-type: none"> •Blocked pro-survival signals from the microenvironment. •Induces apoptosis. •Causes lymphocytosis.
	PI3K p110 δ isoform inhibitors	Idelalisib	CAL-101 GS1101	<ul style="list-style-type: none"> •Blocked pro-survival signals from the microenvironment. •Induces apoptosis. •Increases egress from lymph node by increasing S1PR1 expression.
	PI3K p110 δ / γ dual inhibitors	Duvelisib	IPI-145, INK1197	<ul style="list-style-type: none"> •Blocks pro-survival signals from the microenvironment. •Induces apoptosis. •Causes lymphocytosis.
Immunomodulatory drugs		Lenalidomide		<ul style="list-style-type: none"> •Cereblon (CBRN) decreases Ikaros and Aiolos transcription factors. Tissue Microenvironment •Effect on T cells •Decreases CLL cell proliferation
BCL-2 inhibitors	BH3 mimetic	ABT-199		<ul style="list-style-type: none"> •Selective to BCL-2 •Potently induces apoptosis in CLL cells but not platelets

1.1.5.1.2 Fludarabine and cyclophosphamide (FC)

Fludarabine (F), a purine nucleoside analogue, was first used to treat CLL in the 1980s and demonstrated marked cytoreductive activity (Keating et al., 1989). Fludarabine is a fluorinated analogue of adenine and, upon uptake by a cell, is converted into fludarabine triphosphate which is then incorporated into DNA, resulting in inhibition of DNA and/or RNA synthesis (Adkins et al., 1997). Fludarabine triphosphate also directly inhibits activity of ribonucleotide reductase and DNA polymerase, thereby inhibiting DNA synthesis and repair (Pettitt, 2003). Consequently, it potently induces death of CLL cells by activating the mitochondrial apoptosis pathway (Robertson et al., 1993, Genini et al., 2000). Of note, fludarabine-induced apoptosis can be mediated by p53-dependent and -independent mechanisms (Pettitt et al., 1999a, Pettitt et al., 1999b).

Cyclophosphamide (C) is an alkylating agent, which, like chlorambucil, causes DNA damage by inducing DNA interstrand cross-links. Addition of fludarabine inhibits the cellular process of DNA repair, resulting in enhanced cytotoxicity in CLL cells (Yamauchi et al., 2001). Three randomised trials have shown that fludarabine and cyclophosphamide combination chemotherapy improves the complete response (CR), overall response (OR) and progression free survival (PFS) as compared to fludarabine monotherapy (Eichhorst et al., 2006, Flinn et al., 2007, Catovsky et al., 2007).

1.1.5.1.3 Bendamustine

Bendamustine has been licensed for use in CLL since 2008 as it performed better than chlorambucil in a phase III trial where overall response rates were 68% for bendamustine as compared to 31% for chlorambucil (Knauf et al., 2009).

Bendamustine is chemically related to alkylating agents and induces intrastrand and interstrand cross-links between DNA bases, resulting in inhibition of DNA replication, repair and transcription (Gandhi and Burger, 2009). There is some evidence suggesting that additional or unique mechanisms are at work with bendamustine since there is incomplete cross-resistance between bendamustine and other alkylating agents (Leoni et al., 2008).

Bendamustine (B) in combination with rituximab (R) has been shown to be effective in patients with relapsed and/or refractory CLL (Fischer et al., 2011) and for previously untreated patients with CLL (Fischer et al., 2012). However, a very recent clinical study has indicated that BR is not as effective as FCR in previously untreated and physically fit patients with advanced CLL (Eichhorst, 2014). However, bendamustine is generally better tolerated by less fit patients compared to FC, and thus is currently used in combination with Rituximab to treat this group.

1.1.5.2 Immunochemotherapy

Immunochemotherapy combines chemotherapy with an antibody therapy.

Immunochemotherapy consisting of fludarabine (F), cyclophosphamide (C) and rituximab (R) is now a standard first-line therapy for fit CLL patients. The addition of R to FC improved progression-free survival (PFS) and the overall survival (OS) of patients with CLL (Hallek et al., 2010, Fischer, 2012).

Immunochemotherapy is likely to remain the gold standard therapy in first-line treatment for the foreseeable future (Delgado et al., 2014, Morabito et al., 2015).

1.1.5.2.1 Anti-CD20 antibodies

CD20 is a surface marker expressed on mature B cells and most malignant B cells. CD20 is a good target for immunotherapy since it is expressed at high levels on most B cell malignancies and is not internalised or shed from the plasma membrane following antibody binding (Glennie et al., 2007). This property enables an antibody to remain on the cell surface for a prolonged period, which provides an opportunity for sustained immunological attack from complement and Fc receptor-expressing immune cells such as macrophages.

Anti-CD20 antibodies work either to engage effector pathways, i.e. complement dependant cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP) or by directly inducing apoptosis. All anti-CD20 monoclonal antibodies (mAbs) induce antibody dependent cellular cytotoxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP) which utilises immune cells bearing Fc receptors. Fc binding to Fc γ receptors expressed on monocytes, macrophages, natural killer (NK) cells and neutrophils can lead not only to ADCC and ADCP activities, but also direct killing due to hypercrosslinking. In

antibody-dependent cell-mediated-cytotoxicity (ADCC), rituximab recruits effector cells by binding to their FC γ receptors and this triggers effector cells to release of pre-forming proteins and proteases thus resulting in target cell death. In antibody-dependent cellular phagocytosis (ADCP) rituximab recruits monocytes/macrophages by binding to their FC γ receptors and this results in engulfment of antibody coated tumour cells (Oflazoglu and Audoly, 2010, Okroj et al., 2013).

Preclinical murine models suggest that CD20 antibodies are classified into two types: type I which cluster CD20 into lipid microdomains and potentially activate complement dependent cytotoxicity (CDC) but do not induce apoptosis directly, and type II which do not cause relocation of CD20 and therefore do not activate CDC, but are strong inducers of direct apoptosis and more ADCC. The two types of antibodies had comparable ADCP (Okroj et al., 2013, Herter et al., 2013).

Rituximab, a type I mAb, is the most commonly used monoclonal CD20 antibody in CLL, either in combination with FC (Hallek et al., 2010, Fischer, 2012) or as combination with chlorambucil (Hillmen et al., 2014). Ofatumumab is another type I CD20 mAb, which is approved for use in combination with chlorambucil in first-line treatment in “unfit” patients (Hillmen et al., 2015) and in patients who are refractory to fludarabine and alemtuzumab (a CD52-specific antibody) (Esencay and Hamad, 2015).

Recently type II CD20 mAbs such as obinutuzumab (GA-101) have been developed and undergone clinical evaluation (Mossner et al., 2010). Obinutuzumab in combination with chlorambucil has shown a clear clinical benefit in elderly and unfit patients with CLL (Goede et al., 2014, Goede et al., 2015b).

A comparison between rituximab, ofatumumab and GA101 in CLL cells *in vitro* showed that both ofatumumab and GA101 were superior to rituximab, but that they worked via different mechanisms of action (Rafiq et al., 2013). GA101 promoted enhanced NK cell activation and ADCC. Ofatumumab elicited superior ADCP with monocyte-derived macrophages, whereas GA101 had reduced activation of monocytes. (Rafiq et al., 2013).

1.1.5.2.2 Other monoclonal antibodies

Alemtuzumab (a CD52-specific antibody) had good effects in early clinical trials of CLL patients, but is no longer commercially available (Esencay and Hamad, 2015).

1.1.5.3 BCR signalling inhibitors

In recent years, there has been a marked progress towards “targeted” therapy in CLL. Given that BCR signalling is important in the pathogenesis of CLL, attention has focused on some key kinases in the BCR signalling pathway, namely PI3K, SYK and BTK (Table 1.7) (Robak and Robak, 2013, Hill et al., 2013). Inhibiting any of these three kinases results in marked improvement in the clinical manifestation of CLL.

Table 1.7: BCR signalling inhibitors

Target	Sub class of drug	Name of drug	Abbreviated/ previous name of drug
BTK	BTK inhibitors	Ibrutinib	PCI-32765
PI3K δ	PI3K p110 δ isoform inhibitors	Idelalisib	CAL-101, GS1101
PI3K δ/γ	PI3K p110 δ/γ dual inhibitors	Duvelisib	IPI-145, INK1197
SYK	SYK inhibitors	Fostamatinib	

The BCR inhibitors were initially trialled on relapsed and refractory CLL patients as monotherapies and were found to be safe and effective. Given the success of these studies, several agents are now being trialled in combination with immunochemotherapy or with immunotherapies alone, as is the case of ibrutinib with rituximab (Burger et al., 2014). There is also a proposed strategy of using two inhibitors to target dual kinases, for examples combining a SYK inhibitor with the PI3K inhibitor idelalisib in CLL (Burke et al., 2014).

1.1.5.3.1 The BTK inhibitor

Bruton’s tyrosine kinase (BTK) is a key component of BCR signalling. Its expression is largely restricted to B cells and BTK is over-expressed in CLL cells and constitutively active in some cases (Herman et al., 2011a), making it an excellent target for B cell malignancies. The inhibitor of BTK, ibrutinib, covalently binds to amino acid cysteine at 481 (C481) of the catalytically active domain of BTK and irreversibly inhibits activity of the kinase and BCR signalling downstream of BTK (Honigberg et al., 2010). An early pre-clinical study showed that ibrutinib was

effective in inhibiting BCR-dependent signalling and proliferation of CLL cells (Herman et al., 2011a). It also showed that ibrutinib blocked pro-survival signals from the microenvironment including CD40L, BAFF, TNF- α , IL-4 and IL-6, fibronectin engagement and stromal cell contact.

Early-phase clinical studies have shown that ibrutinib has significant therapeutic activity in patients with relapsed and refractory CLL (Advani et al., 2013, Byrd et al., 2013). Later, a multi-centre, phase III clinical study showed that ibrutinib, in comparison with ofatumumab, significantly improved progression-free survival, overall survival and response rate among patients with relapsed and refractory CLL (Byrd et al., 2014).

One clinical feature associated with ibrutinib therapy is that it induces lymphocytosis in most patients treated (Advani et al., 2013, Byrd et al., 2013, Byrd et al., 2014). It is believed that ibrutinib-induced lymphocytosis is caused by redistribution of malignant lymphocytes between different anatomic compartments, most likely due to the disruption of BCR- and chemokine-mediated stromal chemotaxis and adhesion by the inhibitor (de Rooij et al., 2012, Ponader et al., 2012). It has thus been shown that the prolonged lymphocytosis is mainly composed of non-dividing, quiescent leukaemic cells and does not predict poor outcome or relapse (Woyach et al., 2014b). A recent follow-up study of ibrutinib therapy in CLL showed that this lymphocytosis was largely asymptomatic and disappeared with continued treatment with the drug (Byrd et al., 2015). Therefore this lymphocytosis has no impact on ibrutinib treatment and does not represent a risk factor for disease progression.

Meanwhile, treatment acquired resistance to ibrutinib has been reported in six CLL cases (Woyach et al., 2014a, Furman et al., 2014a). Targeted genome sequencing detected novel mutations responsible for the resistance; four patients carrying mutations in the gene encoding for BTK, one with a mutation in PLC γ 2 and one with mutations in both BTK and PLC γ 2. The C481S mutation in BTK prevents ibrutinib from covalently binding to the cysteine residue of BTK and renders the drug ineffective. The R665W mutation at PLC γ 2 is a gain of function mutation, which enables mutated PLC γ 2 to be activated independently of BTK after BCR engagement, thus implying the existence of a novel pathway that bypasses BTK, possibly involving proximal kinases SYK and LYN (Liu et al., 2015b). These

mutations show that the efficacy of ibrutinib is due to an on-target effect and may be exploitable by development of next generation targeted therapies.

The emergence of resistance to ibrutinib therapy, and possibly to other targeted therapies as well, shows clearly the need to inhibit proliferation which drives evolution and the need to destroy residual clones that are often “hidden” in the microenvironment. It has thus been suggested that the use of drug combinations, as opposed to a single agent approach, is desirable in order to limit the avenues that a cancer cell might be able to take to escape the “hit” and survive (Young and Staudt, 2014).

Other BTK inhibitors are also entering early-phase clinical development such as CC-292 (AVL-292) and ONO-4059 (Burger, 2014) and many more are in development (Akinleye et al., 2013).

1.1.5.3.2 PI3K p110 δ isoform inhibitor

Consistent with its essential role in normal B-cell development and functions, the PI3K p110 δ isoform has recently been reported to be overexpressed in CLL cells (Herman et al., 2010, Lannutti et al., 2011), making it a rational therapeutic target for B-cell lymphoproliferative diseases. The novel inhibitor selective to PI3K p110 δ isoform, idelalisib, has thus displayed promising preclinical activity in CLL by potently inhibiting PI3K signalling activated by intrinsic and extrinsic survival factors (Herman et al., 2010, Lannutti et al., 2011).

An early-phase clinical study has shown encouraging results. Idelalisib monotherapy resulted in nodal responses (treatment-induced lymphocytosis) in 81% of patients with relapsed or refractory CLL, together with a 72% overall response and 33% partial response rate (Brown et al., 2014). The median progression-free survival for all patients was 15.8 months. In the same study, it has been shown that idelalisib-mediated inhibition of PI3K δ led to abrogation of AKT phosphorylation in primary CLL cells from patients and significantly reduced serum levels of CLL-related chemokines (CCL3, CCL4, and CXCL13). A phase 3 clinical study applying idelalisib in combination with rituximab versus rituximab alone in unfit patients with relapsed CLL showed overwhelming efficacy at an interim analysis (Furman et al., 2014b). Patients receiving idelalisib, as compared to those who did not, had

improved rates of overall response (81% vs. 13%) and overall survival at 12 months (92% vs. 80%). Thus the combination of idelalisib and rituximab is a suitable treatment for patients with relapsed CLL who are less able to undergo chemotherapy (Furman et al., 2014b).

Regarding molecular mechanisms underlying its impressive clinical activity, idelalisib has been shown to induce apoptosis of CLL cells, but not other normal T or NK (natural killer) cells (Herman et al., 2010, Lannutti et al., 2011). It also potently inhibited PI3K activity in CLL cells stimulated by pro-survival microenvironmental factors. In addition, idelalisib inhibited CLL-cell chemotaxis toward CXCL12 and CXCL13 and migration beneath the stromal cells. It also reduced secretion of chemokines in stromal co-culture and after BCR engagement, thus inhibiting BCR- and chemokine receptor-induced survival (Hoellenriegel et al., 2011). Recently, it has also been shown to interrupt integrin-mediated CLL cell adhesion to endothelial cells and bone marrow stromal cells, thus inhibiting cell adhesion-mediated survival in co-cultured leukaemic cells (Fiorcari et al., 2013). Most recently, idelalisib was shown to increase the expression of egress receptor, sphingosine-1-phosphate (S1P) receptor 1 (S1PR1) leading to increased migration toward S1P particularly in unmutated CLL cases. Idelalisib's ability to increase S1PR1 expression provides one possible molecular mechanism to explain the lymphocytosis observed in patients when idelalisib is administered in the clinic (Till et al., 2015). Taken together, these studies therefore suggest a triple mechanism of action by idelalisib, i.e. to directly induce apoptosis, to inhibit interactions that keep CLL cells alive in the protective tissue microenvironments and to stimulate CLL egress from the lymph nodes.

1.1.5.3.3 PI3K p110 δ / γ dual inhibitor

Duvelisib (IPI-145, INK1197) is a potent, orally bioavailable dual inhibitor of PI3K- δ and PI3K- γ isoforms (Winkler et al., 2013). Duvelisib was shown to be safe in patients with haematological malignancies in phase I trials (Flinn, 2012) and clinical activity was observed in relapsed and refractory CLL patients including those with relapsed or refractory high-risk disease (*TP53* mutations and / or 17pdel) where it induced rapid lymphocytosis resulting in reduced adenopathy (Flinn, 2013). The most recent data shows that duvelisib continues to demonstrate clinical activity, with an overall response rate (ORR) of 55% in a heavily pre-treated relapsed and

refractory CLL population, including patients with poor prognostic features (O'Brien, 2014). These results support the further clinical evaluation of duvelisib monotherapy and a randomized phase 3 study in patients with relapsed and refractory CLL is ongoing (O'Brien, 2014). As duvelisib has been demonstrated to overcome ibrutinib resistance in a model cell line mimicking C481S mutation in BTK (Dong et al., 2014), it is currently being clinically evaluated in CLL patients who have developed ibrutinib resistance (Porcu, 2014). Finally, early analysis of duvelisib administered in combination with bendamustine or rituximab or both has shown that these combinations are generally well tolerated with encouraging results (Flinn, 2014).

1.1.5.3.4 SYK inhibitors, e.g. Fostamatinib

Fostamatinib is a reversible inhibitor of SYK, an essential downstream mediator of BCR signalling in CLL (Gobessi et al., 2009). Preclinical studies showed that fostamatinib inhibits BCR and integrin signalling, abrogates pro-survival effect of stromal cells, reduces migration to chemokines and induces apoptosis of CLL cells (Quiroga et al., 2009, Buchner et al., 2010). Early-phase clinical study showed that fostamatinib has significant clinical activity in patients with relapsed and refractory CLL, achieving an ORR of 54.5% (Friedberg et al., 2010). Subsequent analysis showed that fostamatinib inhibits BCR signal transduction, activation of downstream effector pathways and cell proliferation *in vivo* (Herman et al., 2013). However, the dose-limiting adverse effects of fostamatinib have restricted the ability to achieve maximum efficacy and hence limited its clinical development. Recently, a more potent and selective SYK inhibitor, entospletinib (GS-9973) has been developed (Currie et al., 2014), which is now under clinical evaluation in a multi-centre, phase 2 study in patients with relapsed or refractory CLL (Sharman et al., 2015).

1.1.5.4 Immunomodulatory drugs

Immunomodulatory drugs (IMiDs) (thalidomide, lenalidomide and pomalidomide) have been shown to bind to cereblon (CRBN), an E3 ubiquitin ligase complex constituent, to facilitate the ubiquitination and proteolysis of two transcription factors Ikaros (IKZF1) and Aiolos (IKZF3), which are required for survival of multiple myeloma cells (Keegan and Figg, 2014). As Ikaros and Aiolos are also highly expressed in CLL cells, IMiDs have displayed encouraging clinical activity in CLL as well (Kater et al., 2014).

Lenalidomide has been shown to interfere with signals received from nurse-like cells, mesenchymal stromal cells and endothelial cells, as well as to repair T and NK cell synapse and motility functions in CLL (Kater et al., 2014). Lenalidomide is not directly cytotoxic to CLL cells *in vitro*, but does inhibit CLL-cell proliferation in a cereblon/p21-dependent manner, but independently of p53 (Fecteau et al., 2014).

Clinical studies have shown that lenalidomide as a monotherapy has had partial responses in 72% of previously untreated patients and in 25-30% of patients with fludarabine refractory CLL. Further trials with lenalidomide in CLL are presently being undertaken and it remains to be seen how this drug will eventually be best used in the clinic (Kater et al., 2014).

1.1.5.5 BCL-2 inhibitors

Anti-apoptotic BCL-2 protein is overexpressed in CLL cells, contributing to cell survival and accumulation of leukaemic cells *in vivo*. High levels of BCL-2 are associated with resistance to chemotherapy and shorter overall survival in CLL (Robertson et al., 1996, Faderl et al., 2002), making BCL-2 a rational target for novel therapeutic agents. BCL-2 mediates its function mainly through directly interacting with the multi-domain pro-apoptotic effectors of the BCL-2 family of proteins, keeping them inactive by altering their subcellular localisation or conformation (Youle and Strasser, 2008, Chipuk et al., 2010, Czabotar et al., 2014). One major approach to target BCL-2 is the development of BH3 mimetic compounds, small molecules that mimic the action of the BH3-only proteins, which are the natural inhibitors of BCL-2 (Vogler et al., 2009b, Billard, 2013).

Early versions of BH3 mimetics (such as Navitoclax, also known as ABT-263) targeted both BCL-2 and BCL-XL due to their shared sequence similarity. As the latter is required for platelet survival, development of ABT-263 was halted due to its induction of dose-limiting thrombocytopenia. Note ABT-737 preceded ABT-263, but was not orally bioavailable (Yosef et al., 2016). Venetoclax (ABT-199) is the latest BH3 mimetic that is selective to BCL-2 and potently induces apoptosis in CLL cells but not platelets (Souers et al., 2013). Venetoclax is highly cytotoxic against CLL cells *in vitro* irrespective of functional status of TP53 in these cells (Anderson et al., 2013). Current early-phase clinical study showed that venetoclax displays promising activity in CLL patients who failed to respond to previous therapies. Although it

initially caused tumour lysis syndrome in some patients, it did not lead to drug-related thrombocytopenia (Seymour, 2013). A phase 1 study showed venetoclax to have a manageable safety profile and the ability to induce substantial responses in patients with relapsed CLL, including those with poor prognostic features (Roberts et al., 2016).

1.1.5.6 MCL1 inhibitors

MCL1 is constitutively expressed in CLL cells, and it is more highly expressed in poor prognosis patients and its expression can be used to predict patient clinical outcome (Kitada et al., 1998, Pepper et al., 2008, Awan et al., 2009). In CLL cells *ex vivo*, MCL1 is involved in the resistance to ABT-737 and navitoclax (ABT-263) (van Delft et al., 2006), and silencing MCL1 induces apoptosis (Hussain et al., 2007). Therefore there has been significant attempts at targeting MCL1 in CLL cells (Besbes et al., 2016).

Agents inhibiting MCL1 can be classified into those that are BH3 mimetics and which inhibit MCL1 directly, and others are agents which inhibit MCL1 expression.

Several pan-BCL-2 family inhibitors such as obatoclax, sabutoclax and AT-101 and others have been developed with low micromolar potency against MCL1. BH3 mimetics, particularly the growing field of MCL1 inhibitors, is a huge topic and three reviews can provide readers with further information if needed (Belmar and Fesik, 2015, Balakrishnan and Gandhi, 2013, Besbes et al., 2016).

Obatoclax overcomes resistance to other BH3 mimetics such as ABT-737 *in vitro* (Nguyen et al., 2007), induces apoptosis of CLL cells treated *ex vivo* and was additive with the cytotoxic agents fludarabine and chlorambucil (Campas et al., 2006). However, in a phase I clinical trial for advanced CLL, Obatoclax had limited effect (O'Brien et al., 2009). Sabutoclax (BI97C1), an Apogossypol derivative, and BI112D1, both pan-BCL-2 inhibitors with activity against MCL1 has also been tested in CLL cells with initially promising *in vitro* data. (Wei et al., 2010, Varadarajan et al., 2013a, Varadarajan et al., 2013b, Brumatti and Ekert, 2013).

Other inhibitors (BH3 mimetics) with activity against MCL1 include TW-37 and S1 derivatives and MCL1 inhibitor molecule (MIM-1) (Varadarajan et al., 2013b, Brumatti and Ekert, 2013, Besbes et al., 2016). Recently, A-1210477, a selective

authentic BH3-mimetic MCL1 inhibitor was designed and has promising *in vitro* activity in combination with navitoclax (ABT-263) (Levenson et al., 2015).

Many agents can decrease MCL1 expression leads to apoptosis in CLL cells: to mention, but three examples: homoharringtonine (HHT), sorafenib and spliceostatin A (SSA). Homoharringtonine (HHT) an inhibitor of translation decreased MCL1 expression and thus induce apoptosis in CLL (Chen et al., 2011). Sorafenib is a multikinase inhibitor but also induces apoptosis in CLL cells by rapid translational downregulation of MCL1 (Huber et al., 2011). More recently the spliceosome inhibitor spliceostatin A (SSA) was shown to induce apoptosis in CLL cells, since it altered the splicing of anti-apoptotic MCL-1L to MCL-1s, therefore inhibiting its expression. Spliceostatin in combination with the BCL-2/BCL-xL antagonists ABT-263 or ABT-199 were able to overcome pro-survival effects found in the lymph node and hence it has been proposed that it may be a useful therapy in CLL (Larrayoz et al., 2016).

1.1.5.7 Other therapeutic strategies

The development of the aforementioned new molecular-targeted drugs offers new opportunity to patients who need alternative treatment and could herald a new era of treatment in CLL. Other therapeutic strategies include proteasome inhibitors and HSP90 inhibitors, which have been extensively reviewed elsewhere (Walsby et al., 2012, Hill et al., 2013).

The costs associated with new immunochemotherapies are already high. Older treatments such as six cycles of chlorambucil monotherapy cost US\$2,038 in 2010, whereas one regime of frontline FCR cost US\$43,599, whilst BR was even more expensive at US\$83,094 per regimen (Shanafelt et al., 2010). Although the development of novel compounds such as Ibrutinib and Idelalisib represent significant treatment advances, the high prices that will be charged for their use may undermine the potential promise of these agents by limiting access and reducing adherence (Shanafelt et al., 2015).

Finally, the fact that all patients receiving treatment eventually relapse means that there is always a need for new therapies. Also, there are two major unmet clinical needs for CLL; firstly those patients with chromosomal deletion in 17p and secondly

those who are less fit or who have comorbidities and cannot tolerate the toxicities of chemotherapies. Whether and how the new therapies in development can address these unmet needs will therefore be both a challenge and opportunity for all scientists and clinicians involved in CLL research and treatment.

1.2 Phosphoinositide-3-kinase (PI3K)

Phosphoinositide-3-kinases (PI3Ks) are a family of lipid kinases that integrate signals from growth factors, cytokines and other extracellular stimuli. They play an essential role in mediating many physiologically important cellular functions and/or processes including cell proliferation, growth, survival, motility and metabolism (Di Paolo and De Camilli, 2006, Vanhaesebroeck et al., 2012, Shanware et al., 2013). PI3Ks phosphorylate the 3-hydroxy group of phosphatidylinositol and related inositol phospholipids to generate 3-phosphoinositides at the nearby membrane sites. These lipids then recruit cytoplasmic proteins to assemble signalling complexes, thereby initiating a cascade of signalling events in the PI3K pathways (Cantley, 2002).

All PI3K catalytic subunits have a PI3K core structure consisting of a C2 domain, a helical domain and a catalytic domain. PI3Ks can be subdivided into three classes based on their lipid preference, cellular localization and structure. Class I PI3Ks use phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) as a substrate to generate PtdIns(3,4,5)P₃. Class II PI3Ks are also known as PI3K-C2 kinases as they are larger monomeric enzymes with a carboxy-terminal C2 domain. To date, the lipid substrates of the Class II PI3Ks are still not well characterised, but possibly include PtdIns and PtdIns(4)P, producing PtdIns(3)P and PtdIns(3,4)P₂. Class III PI3K contains only one PtdIns-specific enzyme Vps34 (vacuolar protein sorting 34), which phosphorylates the hydroxy group of phosphatidylinositol to produce PtdIns(3)P (Bunney and Katan, 2010, Vanhaesebroeck et al., 2012, Thorpe et al., 2015).

Due to the extremely large volume of scientific publications on PI3Ks, it is beyond my capacity to review papers describing biological functions of all three classes of PI3Ks. Instead, I will focus on the literature review on biology of class I PI3Ks that are most relevant to the objectives of my PhD study.

1.2.1 Class I PI3Ks

Class I PI3Ks phosphorylate PtdIns(4,5)P₂ to generate PtdIns(3,4,5)P₃.

Phosphatase and tensin homolog (PTEN) reverses this reaction to return

PtdIns(3,4,5)P₃ to PtdIns(4,5)P₂. SHIP phosphatase converts PtdIns(3,4,5)P₃ to PtdIns(3,4)P₂ (Figure 1.8).

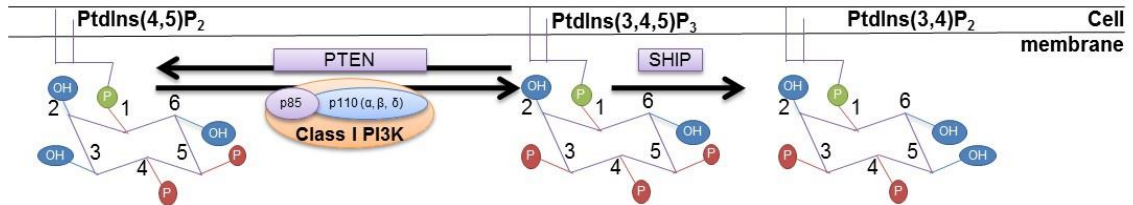


Figure 1.8: Class I PI3Ks phosphorylate PtdIns(4,5)P₂ to generate PtdIns(3,4,5)P₃ at the cell membrane.

Class I PI3Ks phosphorylate PtdIns(4,5)P₂ to generate PtdIns(3,4,5)P₃. Phosphatase and tensin homolog (PTEN) reverse this reaction, by its phosphatase action, to return PtdIns(3,4,5)P₃ to PtdIns(4,5)P₂. SHIP phosphatase turns PtdIns(3,4,5)P₃ to PtdIns(3,4)P₂. Figure based on the review (Vanhaesebroeck et al., 2012).

Class I PI3K enzymes are heterodimeric enzymes composed of a catalytic subunit, the p110 subunit carrying molecular weight of 110 kDa, and a regulatory subunit. They are further divided into subclass IA and IB based on the difference in their regulatory subunits. Class IA is composed of three isoforms of the p110 subunit, namely α, β, δ and the regulatory subunits including p85α, p55α and p50α (encoded by *PIK3R1*) or p85β and p55γ (encoded by *PIK3R2*). Class IB is composed of the catalytic subunit p110γ and regulatory subunit p101 or p87 (Figure 1.9A).

Class IA catalytic subunits (p110α, p110β and p110δ) have a p85-binding domain (p85-BD), a RAS-binding domain (RBD), a helical domain and a catalytic domain. The p110γ subunit has an RBD, a helical domain and a catalytic domain (Figure 1.9A).

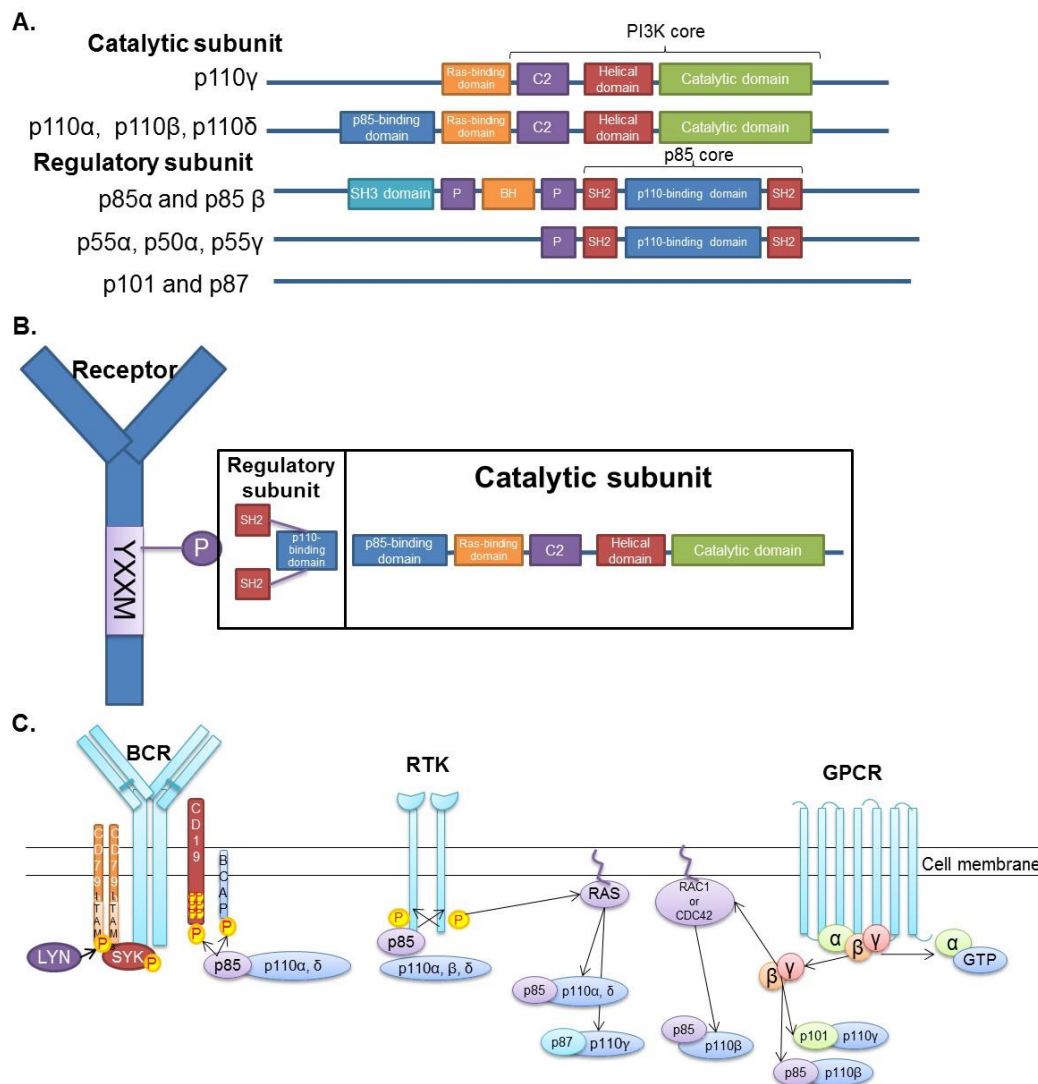


Figure 1.9: Class I PI3Ks.

A. Class I PI3Ks are composed of catalytic and regulatory subunits. Catalytic subunits use phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) as their substrate and exist in complex with a regulatory subunit, either a p85 isoform (for p110 α , p110 β and p110 δ) or p101 or p87 (for p110 γ). Regulatory subunits act to stabilise p110, inactivate p110 kinase activity in the basal state and recruit the PI3K to phospho-tyrosine residues in receptor or adaptor molecules (via SH2 domain, relieves inhibition). The domains of the Class I PI3Ks are shown.

B. Protein-protein interactions of Class I PI3Ks. PI3K p85 regulatory subunit binds to YXXM motif in receptors via SH2 domains. PI3K p110 catalytic subunit binds the regulatory subunit, through the p85-binding domain.

C. PI3Ks are activated by various stimuli: receptor-tyrosine kinases (RTKs), receptor-associated proteins /co-receptors, small GTPases and GPCRs (G-protein coupled receptor small G proteins). All p85 isoforms have SRC homology 2 (SH2) domains which bind to the p-Tyr in a specific amino acid sequence in membrane-associated proteins, either proteins associated with the BCR, CD19 or BCAP thereby bringing p110 α or δ subunits into contact with their lipid substrates. RTKs allow p110 α , β or δ subunits to be activated in a similar manner. Small GTPases (Ras, Rac or CDC42) interact with RTKs and GPCR signals to activate PI3Ks by interacting with their RAS binding domains (RBDs). Isoforms p110 α , p110 δ and p110 γ bind RAS family GTPases, while p110 β binds to the RHO family GTPases RAC and CDC42. G α and G $\beta\gamma$ proteins dissociate from activated G-protein coupled receptors (GPCRs). Catalytic isoforms p110 β and p110 γ , and regulatory isoform p101, directly bind to and are activated by G $\beta\gamma$.

Figure adapted from reviews (Vanhaesebroeck et al., 2012, Vanhaesebroeck et al., 2010, Thorpe et al., 2015).

1.2.1.1 Class I PI3Ks: subunits

All class IA regulatory subunits have two Src homology 2 (SH2) domains and are encoded by *PIK3R1* (which, through differential promoter usage, encodes p85 α , p55 α and p50 α), *PIK3R2* (which encodes p85 β) or *PIK3R3* (which encodes p55 γ). The precise roles of the individual p85 subunits are still unknown. The class IB regulatory subunits p101 and p87 lack SH2 domains and their domain structures are not fully known, but a carboxy-terminal region of p101 has been shown to bind to G $\beta\gamma$ subunits (Vadas et al., 2013). The regulatory subunits are generally less well studied than the catalytic subunits (Figure 1.9A).

PI3K are activated by various stimuli: receptor-tyrosine kinases, receptor-associated proteins/co-receptors, small GTPases and GPCRs (G-protein coupled receptors). As mentioned previously receptor tyrosine kinases can recruit p85 regulatory subunits, allowing for activation of p110 subunits. Regulatory p85 subunits have SRC homology 2 (SH2) domains which bind to the p-Tyr in a specific amino acid sequence (YXXM motifs) in membrane-associated proteins or receptors. The PI3K p110 catalytic subunit binds the regulatory subunit through the p85-binding domain, thereby bringing the subunits into contact with their lipid substrates (Vanhaesebroeck et al., 2012) (Figure 1.9B).

Class IA catalytic subunits (p110 α , p110 β and p110 δ) have a p85-binding domain and a RAS-binding domain (RBD). RTKs can also activate PI3K indirectly via RAS. RAS binds and activates the p110 subunits of PI3K directly (Rodriguez-Viciana et al., 1994, Kodaki et al., 1994). It has been shown that RAS can activate p110 α (Gupta et al., 2007) and p110 γ (Suire et al., 2006, Rubio et al., 1997). Interestingly RAS is needed for the activation of p110 γ bound to p87, but not p101 (Kurig et al., 2009). Finally, despite possessing a RAS binding domain, p110 β appears not to be activated by RAS. Instead, p110 β can be activated by RAC1 and CDC42 RHO family GTPases (Fritsch et al., 2013) and there is some evidence that p110 β can be activated by RAB5 (Christoforidis et al., 1999).

G-protein coupled receptors (GPCRs) transmit their signals intracellularly by activation of heterotrimeric G proteins: G $\beta\gamma$ and G α . PI3K p110 γ signals downstream of GPCRs, with regulatory subunit p101 facilitating the activation of p110 γ via G $\beta\gamma$ subunits (Stoyanov et al., 1995, Stephens et al., 1997, Stephens et al., 1994). p110 β

can also be activated by Gβγ subunits through a mechanism that is presently unclear (Guillermet-Guibert et al., 2008). It is proposed that, since PI3K p110β is expressed more broadly than PI3K p110γ, it may provide a conduit for GPCR-linked PI3K signalling in cell types where PI3K p110γ is not expressed (Vanhaesebroeck et al., 2012) (Figure 1.9C).

1.2.1.2 Class I PI3Ks: tissue distribution of expression

In mammalian cells there are four catalytic and five regulatory class I PI3K subunits. PI3K p110α and p110β subunits have broad distribution of expression in tissues, but p110δ and p110γ are restricted to leukocytes (Table 1.8). Other information regarding expression and functions of isoforms can be found from examining the phenotype of knock-out mice (Table 1.9). There is a discrepancy between the phenotypes caused by p110β and p110γ knockout mice and p110β and p110γ kinase-dead mutant mice, which indicates that there must be kinase independent functions of these two p110 isoforms, see [section 1.2.3](#).

p110α, p110β and p110δ seem to exist as obligate heterodimers with a p85 subunit. This interaction is extremely tight, as the complex can withstand high concentrations of salt, urea or detergent (Kazlauskas and Cooper, 1990, Fry et al., 1992). p85α is essential for functional B cell development and proliferation. When p85α and its splice variants p55α and p50α were disrupted, p85α-p55α-p50α^{-/-} mice died within days after birth, with reduced numbers of peripheral mature B cells, and the B cells that developed had diminished proliferative responses (Fruman et al., 1999b).

PI3K is critical for B cell development, B cell antigen presentation and survival (Pauls et al., 2012, Werner et al., 2010, Baracho et al., 2011, Okkenhaug and Vanhaesebroeck, 2003). Mouse studies have been informative of the individual roles of PI3K p110 isoforms especially in B cells. Below I shall specifically discuss the expression and function of the class I PI3K p110 isoforms.

Table 1.8: Tissue distribution of the mammalian PI3K family members

Class	Role	Gene name	PI3K	Chromosome location	Expression
IA	Catalytic	PIK3CA	p110α	3q26.32	Ubiquitous
IA	Catalytic	PIK3CB	p110β	3q22.3	Ubiquitous
IA	Catalytic	PIK3CD	p110δ	1p36.22	Highly enriched in leukocytes. Moderate expression in neurons and cancer cell lines from various origin (including melanoma, breast, colon). Low expression in most other cell types
IB	Catalytic	PIK3CG	p110γ	7q22.3	High in leukocytes, moderate to low expression in most other tissues
IA	Regulatory	PIK3R1	p85α	5q13.1	Ubiquitous, with lowest expression in skeletal muscle
IA	Regulatory	PIK3R1	p55α	5q13.1	Highest expression in brain and muscle, undetectable in most other tissues
IA	Regulatory	PIK3R1	p50α	5q13.1	High in liver, moderate expression in kidney and brain
IA	Regulatory	PIK3R2	p85β	19p13.11	Ubiquitous, with lowest expression in skeletal muscle
IA	Regulatory	PIK3R3	p55γ	1p34.1	High mRNA expression in brain and testis, moderate mRNA expression in most tissues, mRNA not detectable in liver and muscle. Low protein expression in liver, muscle, fat, liver, spleen.
IB	Regulatory	PIK3R5	p101	17p13.1	High in leukocytes
IB	Regulatory	PIK3R6	p84 / p87	17p13.1	High in leukocytes and heart

Taken from Table 1 and Table 2 of review. (Kok et al., 2009).

Table 1.9: Effect of deleting PI3K specific isoforms in mice.

PIK3CA (<i>p110α</i>)	<ul style="list-style-type: none"> • p110α^{-/-}: died at E9.5, proliferation and vascular defects (Bi et al., 1999, Lelievre et al., 2005) • p110αD933A/D933A: died at E10.5, defective vascular development (Foukas et al., 2006, Graupera et al., 2008) • p110αD933A/WT: viable, metabolic and vascular defects (Foukas et al., 2006, Graupera et al., 2008) • p110α with Ras-binding domain disabled: perinatal lethality, defective lymphogenesis (Gupta et al., 2007)
PIK3CB (<i>p110β</i>)	<ul style="list-style-type: none"> • p110β^{-/-}: died at E3.5 (Bi et al., 2002) • p110βdel/del: partial lethality, no phenotype reported (Guillermet-Guibert et al., 2008). • p110β KinaseDead: partial lethality and metabolic phenotype (Ciraolo et al., 2008)
PIK3CD (<i>p110δ</i>)	<ul style="list-style-type: none"> • p110δ^{-/-}: viable, immunological defects (Clayton et al., 2002, Jou et al., 2002, Puri et al., 2004) • p110δD910A/D910A: viable, immunological defects (Ali et al., 2004) (Okkenhaug et al., 2002)
PIK3CG (<i>p110γ</i>)	<ul style="list-style-type: none"> • p110γ^{-/-}: viable, immunological and cardiac defects (Hirsch et al., 2000a, Patrucco et al., 2004, Rodriguez-Borlado et al., 2003) • p110γ KinaseDead: viable, immunological defects (Patrucco et al., 2004)

Taken from a review (Vanhaesebroeck et al., 2010) – Supplementary 1

1.2.1.3 Class I PI3K p110 subunits

1.2.1.3.1 Redundancy between PI3K isoforms

Whilst many isoform specific inhibitors have been generated. There is some redundancy between PI3K isoforms, as PI3K isoforms are able to compensate for each other, as demonstrated by knockout mouse models and inhibitor studies. The best example of this is competition is between p110α and p110β regulation downstream of RTKs (Utermark et al., 2012). p85–p110α and p85–p110β are both able to bind to the same phosphorylated YXXM motifs in RTKs. Knockout of p110α allows p110β to bind, allowing the RTK to still signal, but at a decreased amount (p110α has higher lipid activity than p110β). Conversely knockout of p110β, allows p110α to bind resulting in increased RTK activity. Genetically or pharmacologically inactivated p110α or p110β can still bind to RTKs but cannot signal – therefore reducing RTK output (Utermark et al., 2012). There is also thought to be some redundancy between p110β and p110γ downstream of GPCRs (Guillermet-Guibert et al., 2008). Thus, it can be difficult, via a single knock out or single pharmacological

inhibition alone to determine the function of each isoform since there is some redundancy between isoforms.

1.2.1.3.2 Class I PI3K p110 α

Promoter analysis of *PIK3CA* shows that p110 α expression can be controlled positively by forkhead box O3a (FOXO3a) and NF κ B and negatively by p53 (Hui et al., 2008, Yang et al., 2008, Astanehe et al., 2008).

Functionally, p110 α seems to be important for platelet-derived growth factor (PDGF)-stimulated signalling and actin reorganization (Hooshmand-Rad et al., 2000).

PI3K p110 α amplification or mutation is found in many solid tumours (Samuels et al., 2004, Shayesteh et al., 1999). Somatic PI3K mutations in cancer are found in *PIK3CA* and rarely in the other p110 isoforms. *PIK3CA* mutations are generally missense mutations and localise to ‘hot-spot regions’ such as the kinase domain or the adjoining helical domain (E542K and E545K). Exactly how *PIK3CA* mutations cause p110 α activation is unclear. His1047Arg mutation may make it less signal-dependent, as it has increased access to its lipid substrate in membranes. However *PIK3CA* mutations seem to be neither necessary nor sufficient for full AKT pathway activation (Mandelker et al., 2009, Gymnopoulos et al., 2007).

Regarding its role in B cells, recent animal studies show that either p110 α or p110 δ can mediate tonic signalling from the BCR, but only p110 δ can contribute to antigen-dependent activation of B cells. p110 α , but not p110 β , compensated in the absence of p110 δ to promote early B cell development in the bone marrow and B cell survival in the spleen. In the absence of both p110 α and p110 δ activities, pre-B cells failed to develop into mature B cells (Ramadani et al., 2010).

1.2.1.3.3 Class I PI3K p110 β

Promoter analysis of *PIK3CB* has not been performed. p110 β seems to be important for insulin signalling (Hooshmand-Rad et al., 2000). p110 β knockout mice are unviable at birth, suggesting that p110 β is required for embryo development (Table 1.9). *PIK3CB* gene is amplified in ovarian cancer and *PIK3CB* mRNA is increased in prostate cancer and neuroblastoma (Zhu et al., 2008, Boller et al., 2008). p110 β

is essential in cell growth, metabolism and tumourigenesis (Jia et al., 2008). PTEN might preferentially couple to p110 β under some conditions [reviewed in Supplementary S3 (Vanhaesebroeck et al., 2010)]. There is probably some competition between p110 α and p110 β regulation in RTK signalling as it has been shown that both p85–p110 α and p85–p110 β compete for phosphorylated YXXM sites on activated RTKs [reviewed in (Thorpe et al., 2015)].

1.2.1.3.4 Class I PI3K p110 δ

p110 δ is highly expressed in leukocytes, present at intermediate levels in neurons and low in all other cell types. p110 δ has been implicated in migration. It is aberrantly expressed in some cancers, such as breast and colon (Sawyer et al., 2003). As well as its involvement in signalling, p110 δ has been shown to regulate membrane fission of Golgi carriers for selective cytokine secretion after TNF α stimulation in macrophages (Low et al., 2010). Crystallography studies have begun to shed light on how p110 δ interacts with p85 α (Burke et al., 2011).

Mice lacking p110 δ isoform are viable but have decreased numbers of mature B cells (marginal zone cells) due to a block in pro-B-cell differentiation, and a B1 B-cell deficiency (Clayton et al., 2002, Jou et al., 2002). Mice expressing inactive mutant p110 δ (D910) exhibit impaired antigen receptor signalling in both B and T cells (Okkenhaug et al., 2002). B cells deficient in p110 δ display defects in BCR signalling as p110 δ is required for BCR-mediated calcium-flux, and activation of PLC γ and BTK. p110 δ deficient B cells respond poorly to CD40 stimulation *in vitro* (Clayton et al., 2002, Jou et al., 2002). Mice with p110 δ deficiency in B cells had markedly reduced serum immunoglobulin levels, and reduced formation of germinal centres in the spleen (Clayton et al., 2002, Jou et al., 2002).

Interestingly, knocking out the p110 δ isoform also affects levels of p85 α , p55 α and p50 α protein expression (Clayton et al., 2002).

It has been described that p110 δ contributes to 60% of total PI3K activity in normal B cells whereas p110 α and β only represent the 40% of remaining activity (Bilancio et al., 2006). Studies using mice expressing kinase inactive p110 δ have shown that p110 δ is the isoform mostly involved in signalling through IL-4 and BCR in B cells. Also, BCR and IL-4 signalling in normal B cells can be prevented by a p110 δ

inhibitor, IC87114 (Bilancio et al., 2006). A further study showed that p110 δ is important for responding to BCR clustering, TLR ligands, LPS and CpG DNA and CXCL13 and sphingosine-1-phosphate (S1P).

The *in vivo* production of natural antibodies requires p110 δ , as inhibitors selective to p110 δ can reduce autoantibody production. Therefore, p110 δ was shown to be critical for B cell function (Durand et al., 2009).

1.2.1.3.5 Class I PI3K p110 γ

PI3K p110 γ is highly expressed in leukocytes and found at lower levels in other cell types. Promoter analysis of *PIK3CG* showed binding sites for transcription factors c/EBP β and GATA-1 (Hirsch et al., 2000b). p110 γ is up regulated after 8 hours in U937 leukaemia cells with all-trans retinoic acid (Baier et al., 1999). Some ovarian cancers have increased *PIK3CG* copy numbers (Zhang et al., 2007). The regulatory subunit p101 facilitates the activation of p110 γ via G $\beta\gamma$ subunits that are exposed from GPCR activation (Stephens et al., 1997).

As far as B cells are concerned, animal studies showed that both p110 γ and p110 δ are expressed in B cells at early developmental stages (Beer-Hammer et al., 2010). They also provided novel evidence that p110 γ and p110 δ have overlapping roles in the development, peripheral maintenance, and function of B cells. Normal B cell differentiation and development requires both isoforms, as p110 γ /p110 δ double deficiency causes an increased percentage of pre-pro B cells and reduced splenic B cell numbers as compared with mice with single deficiency. Functionally, LPS stimulation of splenocytes revealed proliferation defects resulting in decreased survival of B cells in p110 γ /p110 δ double-deficient mice, which correlated with reduced induction of D-type cyclins (required for proliferation) and BCL-XL (required for survival) (Beer-Hammer et al., 2010).

1.2.1.3.6 PI3K p110 δ splice variant p37 δ

A splice variant of p110 δ , p37 δ , was recently reported. Alternative splicing of *PIK3CD* transcripts results in p37 δ , which disrupts the RAS-binding domain and creates a unique C-terminal region. p37 δ was reported to increase cell proliferation and to be overexpressed in human ovarian and colorectal tumours (Fransson et al.,

2012) and has also be found to correlate with poor prognosis in neuroblastoma patients (Fransson and Ejleskar, 2013).

1.2.2 Negative regulators of PI3Ks

As depicted earlier in the Figure 1.8 class I PI3Ks phosphorylate $\text{PtdIns}(4,5)\text{P}_2$ to generate $\text{PtdIns}(3,4,5)\text{P}_3$. This process is negatively regulated primarily by two phosphatases: PTEN and SHIP.

1.2.2.1 PTEN

Phosphatase and tensin homolog (PTEN) is a phosphatase made up of 403 amino acids that can mediate dephosphorylation of substrates such as lipids and peptides. PTEN is located at chromosome 10q23 and is frequently mutated (Chalhoub and Baker, 2009). PTEN has five domains that are important for its activity: a basic N terminus, a phosphatase domain, a C2 domain, a carboxyterminal tail and a PDZ-binding domain. PTEN is an important negative regulator in the PI3K pathway as it counteracts PI3K by converting $\text{PI}(3,4,5)\text{P}_3$ to $\text{PI}(4,5)\text{P}_2$, thereby decreasing the activity of PI3K (Hopkins et al., 2014). In many human cancers PTEN is either mutated or its expression is decreased, leading to increased PIP_3 and therefore increased AKT signalling (Song et al., 2012) (Figure 1.8). Knockdown of PTEN in the haematopoietic compartment of an RNA interference based transgenic mouse model after birth resulted in highly disseminated T-cell acute lymphoblastic leukaemia. Notably, reactivation of PTEN mainly reduced T-cell leukaemia dissemination but had little effect on tumour load in haematopoietic organs, showing that PTEN action is dictated by the tissue microenvironment (Miething et al., 2014).

1.2.2.2 SHIP

SHIP (SH2-containing Inositol 5-Phosphatase) dephosphorylate $\text{PI}(3,4,5)\text{P}_3$ to generate $\text{PI}(3,4)\text{P}_2$ (Figure 1.8). To date, there are two characterised members of the SHIP family of phosphatase, SHIP1 and SHIP2 (Bunney and Katan, 2010). Expression of SHIP1 is restricted to haematopoietic and spermatogenic cells, whereas SHIP2 is ubiquitously expressed in blood cells, skeletal muscle, heart, placenta and pancreas (Rohrschneider et al., 2000, Bunney and Katan, 2010). Whilst mutation in SHIP phosphatase is not a common event in human cancer, one somatic mutation (V684E) in the phosphatase active site of SHIP1 has been reported in

primary acute myeloid leukaemia cells. The mutation caused decreased activity of SHIP1, resulting in enhanced activity of AKT and survival of the leukaemic cells (Luo et al., 2003).

1.2.3 Scaffolding function of class I PI3Ks

In addition to the many important functions associated with their kinase activity, class I PI3Ks may also act as a scaffold protein to exert their functions and/or influence the activity of other proteins (Costa and Hirsch, 2010, Vanhaesebroeck et al., 2010, Hirsch et al., 2009). For example, it has been shown that PI3K p110 γ negatively regulates heart contractility by binding to phosphodiesterase 3B, a major enzyme involved in cAMP degradation (Patrucco et al., 2004). PI3K p110 γ has also been reported to regulate the oxidative bursts in neutrophils in response to bacterial peptides via direct interaction with PKC α (Lehmann et al., 2009). In addition, PI3K p110 β has been shown to interact directly with proliferating cell nuclear antigen (PCNA), resulting in enhanced loading of PCNA onto chromatin and DNA replication (Marques et al., 2009). Understanding the kinase-independent function of the PI3Ks is important as it helps us to understand whether scaffolding function is required for the optimal activity of the kinase or whether it is independent of the catalytic function of the enzyme. Consequently, this knowledge will impact the therapeutic approach to employ agents that can disrupt protein-protein interaction, together with classical ATP competitors, to synergistically inhibit the kinase-dependent and -independent functions, achieving better efficacy.

1.2.4 Downstream effectors of class I PI3Ks

It is estimated that there are 50-100 downstream effectors of PI3K and approximately 20-50 proteins in a typical mammalian cell that are regulated via direct binding to PIP₃ or PI(3,4)P₂, including PH domain-containing guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) (Vanhaesebroeck et al., 2012) and BAM32 (B-cell adaptor molecule of 32 kDa) (Okkenhaug and Vanhaesebroeck, 2003).

Among the 20-50 proteins downstream of PI3K, AKT is the most important mediator of the PI3K signalling pathway (Manning and Cantley, 2007, Fayard et al., 2010, Hemmings and Restuccia, 2012). I will therefore review the literature on the biology

of AKT in a separate section (see [section 1.3](#) below), but mention briefly PI3K activation of BTK and complete the current section with an update on the current status of preclinical and clinical development of PI3K inhibitors.

1.2.5 PI3K-mediated regulation of BTK

The TEC-family kinases are thought to link PI3K activation to calcium signalling. BTK is a TEC-family kinase expressed in B cells and has a PH domain that binds PIP₃ with high affinity (Okkenhaug and Vanhaesebroeck, 2003). BTK phosphorylates PLC γ , which cleaves PIP₃ to generate DAG and IP₃. At a threshold level of IP₃, Ca²⁺ is released from intracellular stores, causing calcium flux into the cytosol, which is required for a sustained calcium response (Scharenberg et al., 1998) (Figure 1.10). BTK is required for the transcription of a small subset of genes that are not regulated by PI3K, implying BTK can also be activated independently of PI3K (Fruman et al., 2002).

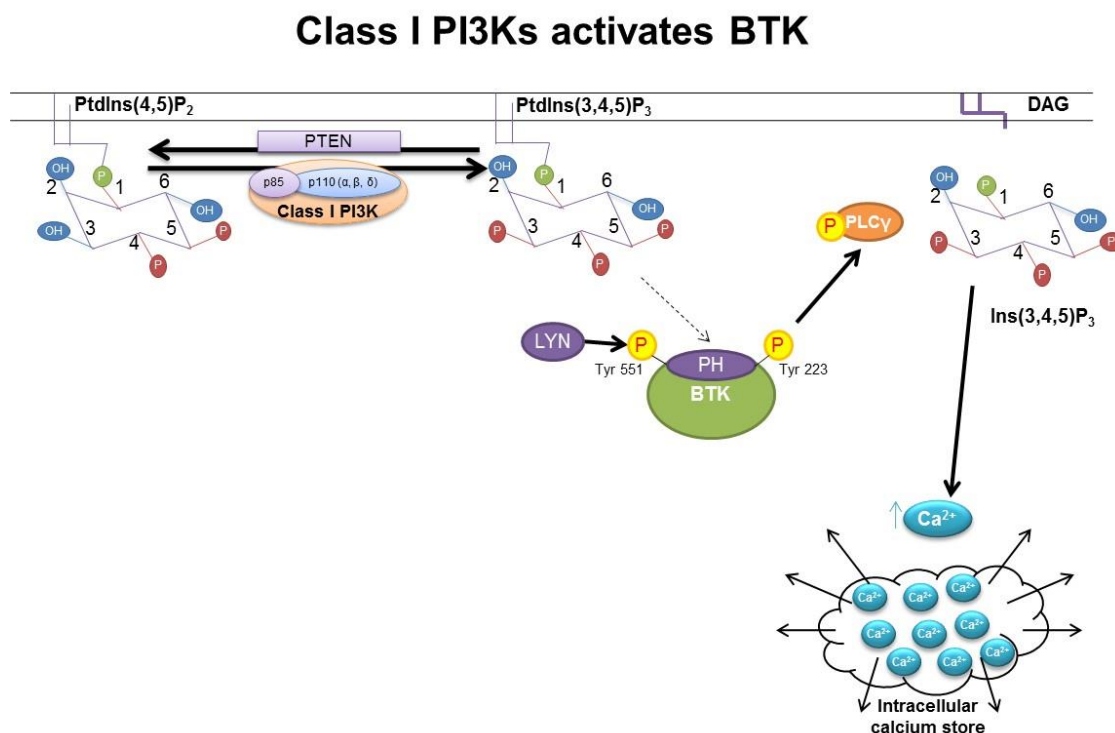


Figure 1.10: Class I PI3K activates BTK.

BTK is a TEC-family kinase expressed in B cells and has a PH domain that binds PIP₃ with high affinity. BTK phosphorylates PLC γ , which cleaves PIP₃ to generate DAG and IP₃. At a threshold level of IP₃, Ca²⁺ is released from intracellular stores, causing a calcium flux into the cytosol, which is required for a sustained calcium response.

1.2.6 Pharmacological inhibitors of PI3Ks

As described earlier, PI3Ks are fundamentally important to many physiological functions of healthy cells and tissues. Altered activity of PI3Ks has been associated with numerous human diseases including diabetes, neurodegenerative diseases, muscle hypotrophy and cancer. Consequently, there has been concerted effort in the scientific community to explore the therapeutic potential of pharmacological inhibitors of PI3Ks for treatment of human diseases, in particular cancers (Rodon et al., 2013, Hennessy et al., 2005, Thorpe et al., 2015, Fruman and Rommel, 2011, Yap et al., 2015).

LY294002 and Wortmannin were the first generation reversible pan-PI3K inhibitors, but these were proven to be too toxic for clinical use (Vlahos et al., 1994, Wymann et al., 1996). More recently, kinase screening has led to the development of the p110 δ inhibitors idelalisib and IC87114 (Lannutti et al., 2011). Further kinase screening and high-throughput and virtual screening will likely lead to the development of more novel PI3K inhibitors in the near future (Zhu et al., 2015). PI3K inhibitors generally fall into three major categories; pan-PI3K inhibitors, dual PI3K/mTOR inhibitors, or PI3K isoform-specific inhibitors. Currently available pan-PI3K and PI3K isoform specific inhibitors are displayed in Table 1.10. Below I summarise in what setting PI3K inhibitors might be useful and their known toxicities.

1.2.6.1 Pan PI3K inhibitors

Pan PI3K inhibitors such as Pictilisib (GDC-0941) cause ‘on-target’ toxicities including rash, fatigue, hyperglycaemia and gastrointestinal symptoms. No pan-PI3K inhibitors have entered clinical development as a single agent.

1.2.6.2 Isoform specific PI3K inhibitors

1.2.6.2.1 PI3K p110 α inhibitors

Screening for *PIK3CA* mutation is useful and partially determines if p110 α inhibitors will be effective. Hyperglycaemia is frequently observed with p110 α inhibitors, as well as fatigue and gastrointestinal symptoms.

1.2.6.2.2 PI3K p110 β inhibitors

There is some evidence that p110 β is responsible for driving the abnormal proliferation of tumour cells harbouring PTEN loss or inactivation and therefore p110 β inhibitors might be specifically useful in this context (Wee et al., 2008, Ni et al., 2012).

1.2.6.2.3 PI3K p110 δ inhibitors

PI3K p110 δ is critical in lymphocyte function (see [section 1.2.1.3.3](#)) and so the PI3K p110 δ inhibitor, idelalisib is now available for the treatment of CLL and other malignancies. The dual p110 δ / γ inhibitor, IPI-145 is in early clinical trials for haematological malignancies. These inhibitors are discussed at length elsewhere (see [section 1.1.5.3.2](#) and [section 1.1.5.3.3](#)). Of note, hyperglycaemia is uncommon with p110 δ / γ inhibitors, but myelosuppression with neutropenia has been reported.

1.2.6.3 Monitoring the activity of PI3K inhibitors

Currently the most common method of ascertaining if PI3K inhibitors are active *in-vivo* is to examine the phosphorylation status of AKT at serine 473 or threonine 308 by immunohistochemistry or Western blotting in PBMCs, hair follicles or tumour samples from patients (Raynaud et al., 2009, Guillard et al., 2009).

PI3K inhibition also reduces the amount of glucose taken up by certain cell types, allowing for another way of monitoring PI3K inhibition, either by measuring blood glucose level and insulin concentrations or by ¹⁸F-fludeoxyglucose (FDG), on a positron emission tomography (PET) (FDG-PET) in tumour tissue (Sarker et al., 2015).

1.2.6.4 Combining PI3K inhibitors

It is likely that PI3K inhibitors will have to be used in combination therapy since resistance mechanisms including feedback and compensatory signalling pathways have been reported when they are used as single agents (Klempner et al., 2013, Rodon et al., 2013). There is cross-talk between the RAS-RAF-MEK pathway and the PI3K pathway, thus forming an escape route to PI3K inhibitors. Therefore combining RAS-RAF-MEK inhibitors with PI3K inhibitors may be prudent (Sos et

al., 2009, Renshaw et al., 2013). Combining PI3K inhibitors with receptor tyrosine kinase inhibitors has also been proposed (Serra et al., 2011).

Table 1.10: Pan-PI3K and Isoform-selective PI3K inhibitor IC₅₀ values.

Based on (Thorpe et al., 2015) and <http://www.selleckchem.com/>. For PI3K inhibitors used in this project see Table 2.11 and for more information regarding these inhibitors, see data from AstraZeneca in Appendix Table 2.

Agent	Company	Isoform target	IC ₅₀ values				References/Additional Information
			p110 α	p110 β	p110 δ	p110 γ	
BKM120	Novartis	Pan-PI3K	52 nM	166 nM	116 nM	262 nM	(Maira et al., 2012, Brachmann et al., 2012)
GDC-0941 (Pictilisib)	Genetech	p110 α / p110 δ	3 nM	3 nM	33 nM	75 nM	(Folkes et al., 2008)
BAY80-6946 (Copanlisib)	Bayer	Pan-PI3K	0.5 nM	3.7 nM	0.7 nM	6.4 nM	(Liu et al., 2013)
ZSTK474	Zenyaku Kogyo Co.	Pan-PI3K	16 nM	44 nM	4.6 nM	49 nM	(Kong and Yamori, 2007)
PX866	Oncothyreon	Pan-PI3K	--	--	--	--	
XL147 (Pilaralisib)	Exelixis / Sanofi-Aventis	Pan-PI3K	39 nM	36 nM	36 nM	23 nM	(Foster et al., 2015)
CH5132799	Chugai Pharma Europe	Pan-PI3K	14 nM	0.12 μ M	0.50 μ M	36 nM	(Tanaka et al., 2011)
BLY719 (Alpelisib)	Novartis	p110 α	5 nM	minimal	minimal	minimal	--
GDC-0032	Genetech	p110 α	0.29 nM (Ki)	9.1 nM (Ki)	0.12 nM (Ki)	0.97 nM (Ki)	(Ndubaku et al., 2013)
INK117 (MLN1117)	Instellikine/Millennium	p110 α	--	--	--	--	--
AZD8186	AstraZeneca	p110 β	35 nM	4 nM	12 nM	675 nM	(Barlaam et al., 2015, Hancox et al., 2015)
TGX-221	--	p110 β	5 μ M	5 nM	0.1 μ M	>10 μ M	(Jackson et al., 2005)
GSK2636771	GlaxSmithKline	p110 β	--	p110β	--	--	
SAR260301	Sanofi	p110 β	--	p110β	--	--	
IPI-145 (INK1197)(Duvelisib)	Infinity	p110 δ / p110 γ	1602 nM	85 nM	2.5 nM	27 nM	(Winkler et al., 2013)
AMG319	Amgen	p110 δ	--	--	p110δ	--	(Cushing et al., 2015)
CAL-101 (GS-1101)(Idelalisib)	Gilead Sciences	p110 δ	820 nM	565 nM	2.5 nM	89 nM	(Lannutti et al., 2011)
GS9820	Gilead Sciences	p110 β / p110 δ	--	p110β	p110δ	--	

1.3 AKT

AKT, also known as Protein Kinase B (PKB), was identified by three independent groups in 1991 (Bellacosa et al., 1991, Coffey and Woodgett, 1991, Jones et al., 1991b). It is a serine-threonine kinase that is indirectly activated by PI3K. There are three isoforms of AKT: AKT1, AKT2, and AKT3. Each isoform has differing but overlapping substrate specificity and cellular localisations. AKT phosphorylates many downstream substrates, resulting in many biological effects including cell growth, metabolism, proliferation and survival. As such, AKT has been identified as a potential drug target (Workman, 2005).

1.3.1 AKT is an AGC kinase

The human kinome can be subdivided into eight groups based on the homology of catalytic domains. AKT is an AGC kinase (Figure 1.11) (Manning et al., 2002b). AGC kinases are named after three representative families, cAMP-dependent protein kinase (PKA), the cGMP-dependent protein kinase (PKG), and the protein kinase C (PKC). Most AGC kinases contain a regulatory PIF (PDK1 Interacting Fragment)-pocket, that serves as a docking site for substrates of phosphoinositide-dependent kinase 1 (PDK1). This pocket is also essential for them to be phosphorylated and activated (Arencibia et al., 2013).

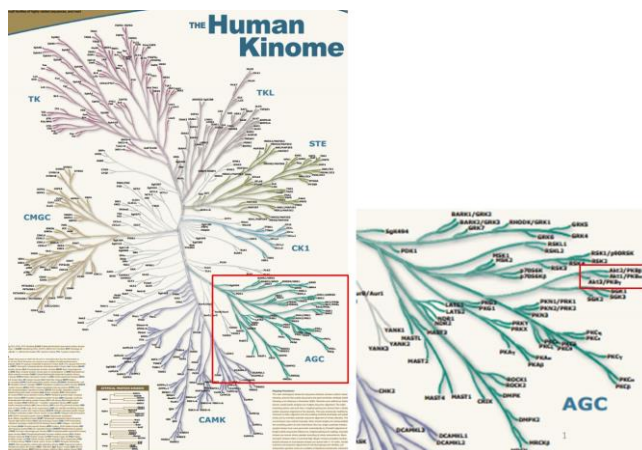


Figure 1.11: AKT are AGC kinases, their location within the human kinome map.

This dendrogram shows the sequence similarities between protein kinases, derived from public sequence databases and gene prediction methods. Many branch lengths are semi-quantitative, but the branching pattern is more informative. AKT1, 2 and 3 are AGC kinases and have similar sequences to other AGC kinases (Manning et al., 2002b).

1.3.2 Regulation of activation of AKT

AKT is normally kept in an inactive state through an intramolecular interaction between the pleckstrin homology (PH) and kinase domains (Fayard et al., 2010, Hers et al., 2011).

1.3.2.1 Activation of AKT by PI3K

Phosphoinositide-3-kinase catalyses $PI(4,5)P_2$ to $PI(3,4,5)P_3$ (PIP_3) allowing the latter to recruit PH domain-containing proteins to the plasma membrane, such as phosphoinositide-dependent kinase 1 (PDK1) and AKT. The PH domain consists of approximately 120 amino acids and bind to phosphoinositols, PIP_3 , $PI(3,4)P_2$, and sometimes $PI(4,5)P_2$ (Harlan et al., 1994, Lemmon, 2007). The PH domains in BTK and GRP1 bind PIP_3 , whereas AKT, DAPP1, and the C-terminal domain of TAPP1 all bind $PI(3,4)P_2$ and PIP_3 (Ananthanarayanan et al., 2005). With the generation of PIP_3 , AKT is transiently localised to the plasma membrane by its PH domain (Andjelkovic et al., 1997).

It is now generally accepted that full activation of AKT requires phosphorylation of both threonine 308 by PDK1 and serine 473 by mTORC2 (Figure 1.12). It has been shown that AKT is first phosphorylated at serine 473 prior to phosphorylation at threonine 308 by PDK1 (Sarbasov et al., 2005).

Although mTORC2 is now established as the kinase which phosphorylates AKT at S473 (Stephens et al., 1998), there were previous studies reporting other kinases to be responsible. They included ILK (Delcommenne et al., 1998, Persad et al., 2001) and $PKC\beta II$ (Kawakami et al., 2004). $PKC\beta II$ was required for AKT phosphorylation on S473 only in certain cells and under certain stimuli, such as IgE/antigen-stimulated mast cells, but not antigen-stimulated T or B lymphocytes (Kawakami et al., 2004). It has also been shown that AKT can auto-phosphorylate itself at serine 473 (Toker and Newton, 2000).

$PI(3,4,5)$ -dependent protein kinase-1 (PDK-1) was identified as the kinase that phosphorylates AKT at Thr308 (Alessi et al., 1997). Fluorescent resonance energy transfer (FRET) showed that AKT phosphorylation at Thr308 leads to a conformational change within AKT, resulting in the loss of membrane binding affinity of N-terminal PH-domain, allowing it to detach from the membrane

(Ananthanarayanan et al., 2007). Phosphorylated AKT can then translocate from the plasma membrane to the cytoplasm or nucleus, where it mediates its downstream effects.

TCL1 family proteins form heterodimers with AKT and promote AKT dimerization, and therefore promote AKT activation (Teitell, 2005), see [section 1.3.3.5](#), for further information.

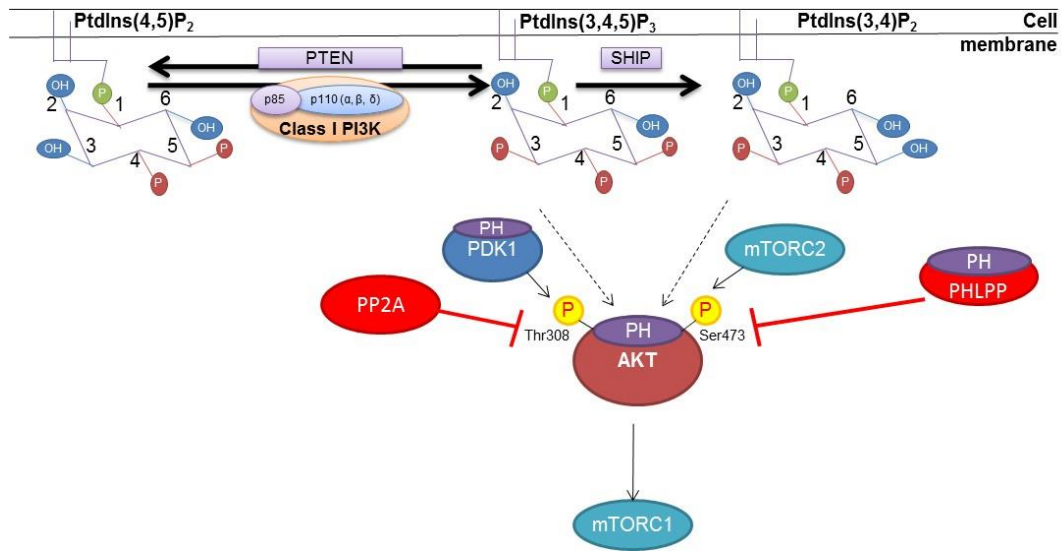


Figure 1.12: Class I PI3Ks activate AKT

Receptor-stimulated class I phosphoinositide 3-kinases (PI3Ks) generate phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃) and, indirectly, PtdIns(3,4)P₂, which bind directly to the pleckstrin homology (PH) domain of AKT, driving translocation of AKT to the plasma membrane and allowing its phosphorylation on Thr308 by phosphoinositide-dependent kinase 1 (PDK1). Additional phosphorylation on Ser473 by mammalian target of rapamycin complex 2 (mTORC2) leads to full activation. Once fully active, AKT drives activation of mTORC1. AKT can be dephosphorylated at Thr308 and at Ser473 by PP2A, or at Ser473 by PHLPP. Figure based on review by (Vanhaesebroeck et al., 2012, Bayascas and Alessi, 2005).

1.3.2.2 Inactivation of AKT

Activated AKT is eventually dephosphorylated by phosphatases to return to its inactive state (Meier et al., 1997, Meier et al., 1998, Meier and Hemmings, 1999).

An early study showed that protein phosphatase 2A (PP2A) can dephosphorylate AKT *in-vitro* and that addition of the PP2A inhibitor okadaic acid can restore phosphorylation of AKT (Andjelkovic et al., 1996). It was later shown that PP2A can cause dephosphorylation of both T308 and S473 residues on AKT in intact cells (Meier et al., 1998).

Later, it was shown that a PH domain leucine-rich repeat protein phosphatase (PHLPP) can dephosphorylate AKT on S473 (Gao et al., 2005). PHLPP is the only protein phosphatase in the human genome possessing a PH domain. The PHLPP family is composed of two genes: *PHLPP1* and *PHLPP2*. *PHLPP1* can be spliced to generate *PHLPP1 α* or *PHLPP1 β* , which are identical except that *PHLPP1 β* contains a 512-amino-acid N-terminal extension that carries a Ras-association domain. All three PHLPPs can dephosphorylate all three AKT isoforms, though with some differential specificities. In light of the newly discovered role of PHLPP in terminating AKT signalling, there are attempts to use PHLPP as a drug target to turn off AKT for therapeutic gain (Newton and Trotman, 2014).

1.3.2.3 AKT protein stability

The half-life of AKT protein has been determined in breast cancer cell lines to be about 36 hours (Basso et al., 2002). AKT has a long half-life as it forms a complex with HSP90 and CDC37 in cells. HSP90 inhibitors have been shown to significantly reduce the half-life of AKT and thus can inhibit its activity in animal models of human breast cancer (Solit et al., 2003).

1.3.2.4 Ubiquitination of AKT

AKT can be ubiquitinated by E3 ligases in order to target it for activation, for degradation, and hence terminate signalling, or to traffic it to subcellular locations with the cells.

TRAF6 E3 ligase orchestrates IGF-1 mediated AKT ubiquitination and activation. AKT is recruited to the plasma membrane by TRAF6 lysine-63 chain ubiquitination at K8 and K14 within the PH domain (Yang et al., 2009). Skp2-SCF is a similarly critical E3 ligase for ErbB-receptor mediated AKT ubiquitination and membrane recruitment following EGF stimulation (Chan et al., 2012).

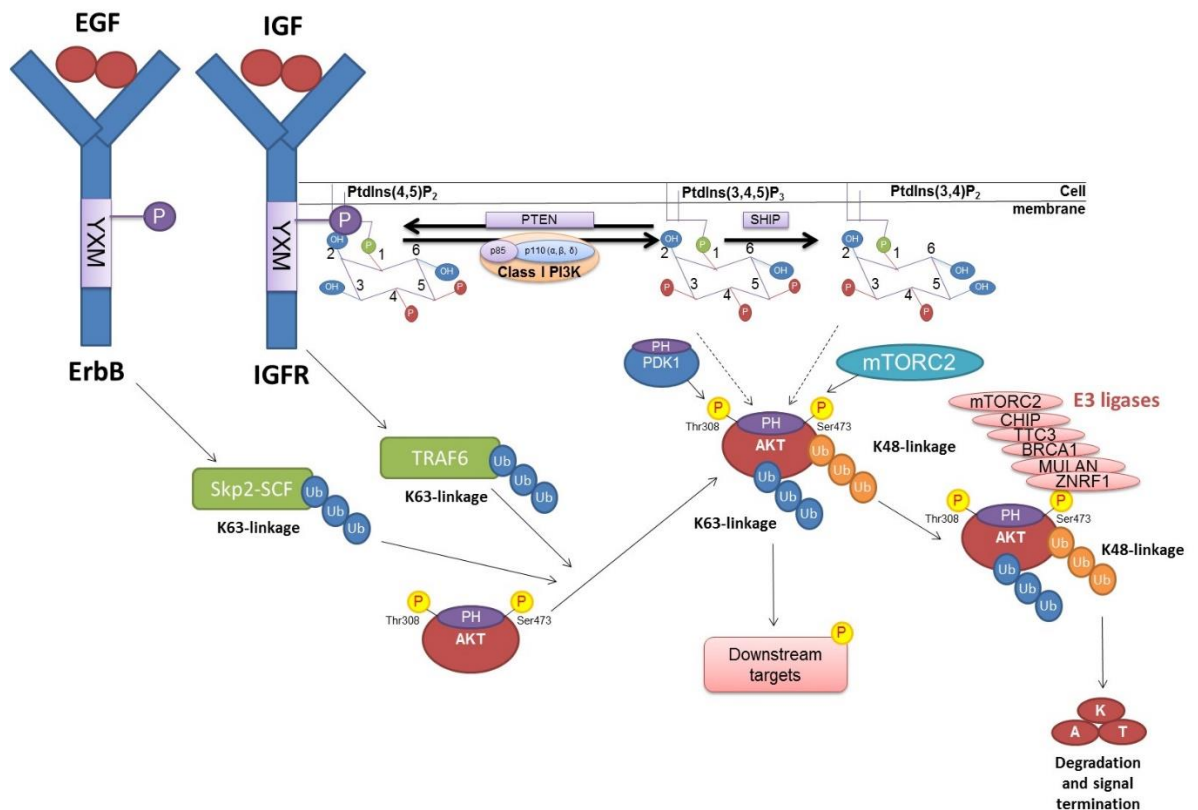


Figure 1.13: Ubiquitination of AKT

IGF-1 induced activation of IGF-1R promotes TRAF6 activation, which then triggers K63-linked ubiquitination of AKT and promotes AKT membrane recruitment. Similarly EGF stimulation of ErbB results in Skp2-SCF recruitment and which then triggers K63-linked ubiquitination of AKT and promotes AKT membrane recruitment. Subsequent phosphorylation by PDK1 and mTORC2. Active AKT then phosphorylates its substrates in the cytoplasm and nucleus to exert its biological effects. E3 ligases, mTORC2, CHIP, TTC3, BRCA1m MULAN and ZNRF1 then target AKT for degradation by the ubiquitin proteasome system. Figure based on reviews by Wang et al., 2012 and Yang et al., 2010a and 2010b. (Wang et al., 2012a, Yang et al., 2010b, Yang et al., 2010a).

AKT ubiquitination by several E3 ligases has been shown to regulate AKT degradation, such as CHIP (carboxyl terminus of Hsc-70 interacting protein), BRCA1, and TTC3 (Dickey et al., 2008, Xiang et al., 2008, Suizu et al., 2009). mTORC2 mediated phosphorylation of T450 also increases AKT stability (Facchinetti et al., 2008).

The AKT Ser-473 phosphorylation by mTORC2 promotes Lys-48-linked polyubiquitination of AKT, resulting in its rapid proteasomal degradation, which is an important negative feedback regulation that specifically terminates AKT activation (Wu et al., 2011). AKT1 and AKT2 can be targeted for degradation by MULAN (which possesses both a RING finger domain and E3 ubiquitin ligase activity) to suppress cell proliferation and viability (Bae et al., 2012). MULAN preferentially binds to phosphorylated AKT, by exposed sites within the AKT kinase

domain (not the phosphorylated residues themselves) to promote AKT's degradation. ZNRF1, an E3 ligase targets AKT to degrade through the ubiquitin proteasome system (Wakatsuki et al., 2011).

NEDD4-1 is an E3 ligase that specifically regulated ubiquitin-dependant trafficking of p-AKT in response to insulin-like growth factor (IGF)-1 signalling. It catalyses K-63 type polyubiquitin chain formation of AKT to translocate AKT to perinuclear region, where it is released into the cytoplasm, imported into the nucleus or coupled with protein degradation (Fan et al., 2013).

The topic of AKT ubiquitination is further reviewed by the following three reviews (Wang et al., 2012a, Yang et al., 2010b, Yang et al., 2010a).

1.3.2.5 PI3K-independent AKT activation

There is evidence suggesting that AKT can be activated independently of PI3K. Recently, some non-receptor tyrosine kinases (e.g. TNK2, Scr and PTK6) as well as some serine/threonine kinases (e.g. TBK1, IKBKE, DNA-PKcs) have been shown to activate AKT in a PI3K-independent manner (Mahajan and Mahajan, 2012).

Recently, AKT activity was shown to fluctuate across the cell cycle and cyclin A2 was shown to phosphorylate AKT at S477 and T479 (Liu et al., 2014), indicating that cyclin A2 has a role in governing AKT activation. The authors also showed that phosphorylation of S477/pT479 of AKT1 can be mediated by CDK2/cyclin A, mTORC2 or DNA-PK under cell cycle progression, growth factor stimulation or DNA damaging conditions, respectively (Liu et al., 2014).

1.3.3 AKT isoforms

1.3.3.1 AKT isoform structure

In mammalian cells, there are three isoforms of AKT (AKT1, AKT2, AKT3) encoded by three different genes, on three different chromosomes. The three isoforms share more than 76% amino acid sequence identity at the PH-domain, and greater than 87% homology in the catalytic domain (Figure 1.14).

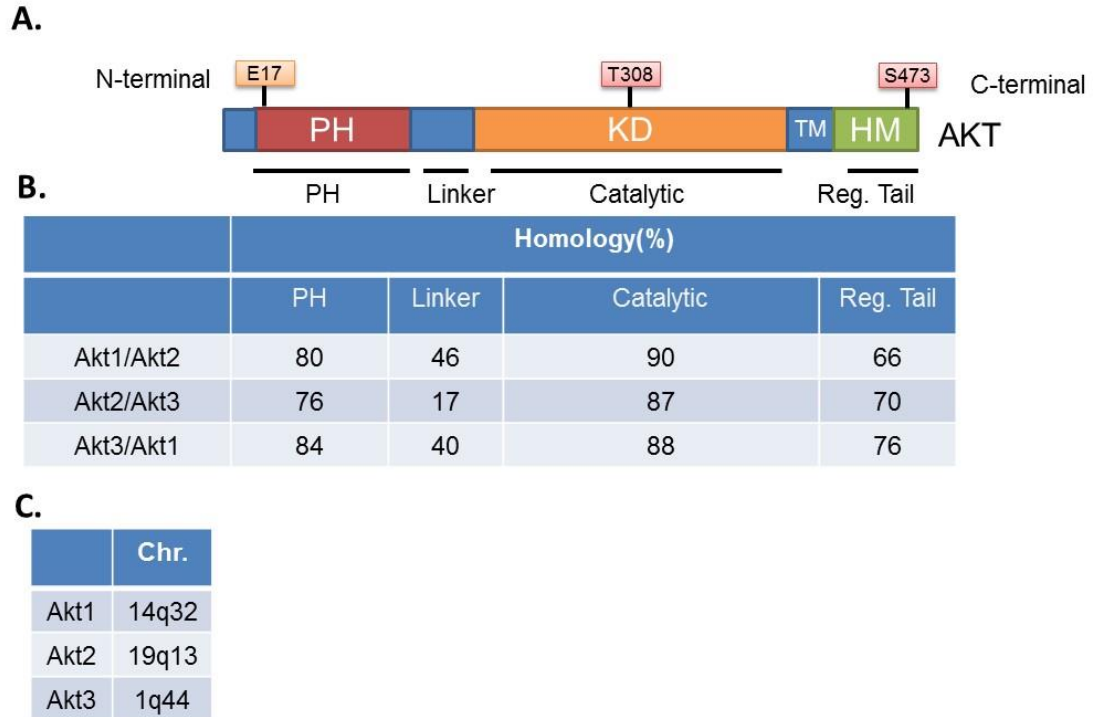


Figure 1.14: Structure of the AKT isoforms.

Based on figure 1. and table 1: Pairwise % identity in AKT domains (Kumar and Madison, 2005) and <http://www.chemdiv.com/portfolio/akt-targeted-library/>

- (A) AKT domains as well as regulatory phosphorylation sites in AKT1 are depicted.
- (B) Comparison of AKT isoforms (% of homology).
- (C) Chromosome location of each AKT isoform in human.

1.3.3.2 AKT isoform functions

Evidence regarding isoform expression and function can be gathered from three main sources: normal tissue distribution, mutations or overexpression of specific AKT isoforms in cancers and finally knockout mice studies (Scheid and Woodgett, 2001, Gonzalez and McGraw, 2009, Lawlor and Alessi, 2001). Recent phosphoproteomic studies also identified AKT isoform specific substrates (Sanidas et al., 2014).

AKT1 is ubiquitously highly expressed in most tissues with the exception of kidneys, liver and spleen (Coffer and Woodgett, 1991, Jones et al., 1991b, Bellacosa et al., 1993). The level of AKT2 expression is highest in muscles and other insulin-sensitive tissues such as liver and adipose. AKT2 expression is also high in the intestines and reproductive tissues (Jones et al., 1991a, Konishi et al., 1994). AKT3 is highly expressed in the brain and testes (Nakatani et al., 1999a).

Various AKT isoforms have been found to be amplified in certain human cancers. AKT1 gene amplification has been found in gastric, pancreatic, ovarian and head & neck cancer (Staal, 1987) and AKT2 gene amplification is frequently observed in ovarian, breast and pancreatic cancer (Staal, 1987, Bellacosa et al., 1995, Cheng et al., 1992, Cheng et al., 1996). AKT3 gene amplification is found in melanomas (Stahl et al., 2004, Carpten et al., 2007).

Overexpression of AKT isoforms at the protein level can also be found in certain tumours. AKT1 is up-regulated in breast cancer (Stal et al., 2003). AKT2 is increased in hepatocellular and colorectal cancer (Roy et al., 2002, Xu et al., 2004) and AKT3 is up-regulated in ER negative breast cancer and some ovarian tumours (Nakatani et al., 1999b, Stahl et al., 2004).

Finally, a mutation in the PH domain of AKT1 (E17K) which results in its constitutive activation, has been found in human ovarian, breast and colorectal cancer (Carpten et al., 2007). An equivalent mutation in AKT3 has also been described in human melanomas (Davies et al., 2008). AKT inhibitors can specifically inhibit the growth of cancer models with an AKT1 E17K mutation. Moreover, treatment with the catalytic AKT inhibitor AZD5363 induced prolonged partial responses in two women with breast and ovarian cancer with AKT1 (E17K) mutations (Davies et al., 2015).

AKT1 knockout mice are viable, but have retarded growth (Chen et al., 2001, Cho et al., 2001b). AKT2 null mice are viable, but have defects in glucose homeostasis as insulin failed to reduce blood glucose levels, implicating AKT2 in insulin signalling (Garofalo et al., 2003, Cho et al., 2001a). AKT3 knockout mice display impaired brain development, implicating AKT3 in neuronal development (Tschopp et al., 2005). Individual knockout of AKT isoforms in mice did not cause lethality suggesting that the three isoforms are able to compensate for one another. However, their unique phenotypes suggest they also have distinct functions. Double knockout studies also support the idea that AKT isoforms are able to compensate for one another. AKT1 and AKT2 are required for postnatal survival as mice deficient in both AKT1 and AKT2 die shortly after birth (Peng et al., 2003). Double knockout of AKT1 and AKT3 is embryonically lethal (Yang et al., 2005), whereas mice deficient in both AKT2 and AKT3 are viable, but have reduced body weight and size

(Dummler et al., 2006). Figure 1.15 summarises the common and specific function of individual isoforms of AKT based on the phenotypic analysis of single and double knockout mice (Gonzalez and McGraw, 2009).

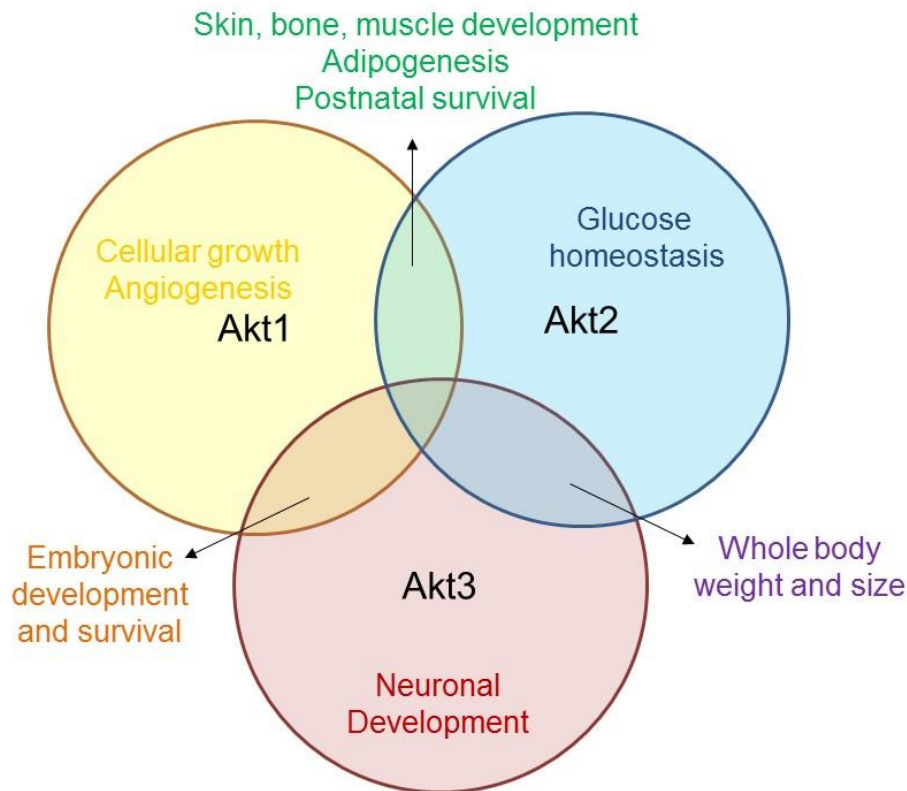


Figure 1.15: Overlapping and specific functions of the AKT family members.

Summarised are the common and specific functions elucidated from the phenotypic analysis of single and double knockout mice. Figure based on figure 1B from review by (Gonzalez and McGraw, 2009)

To examine whether or not the functional differences among the isoforms are due to substrate differences, Sanidas and colleagues performed a phosphoproteomics screen using cell lines expressing each AKT isoform. In total, 606 proteins were discovered that were phosphorylated on AKT consensus sites in at least one of the AKT isoform-expressing cells (Sanidas et al., 2014). Many were already known to lie downstream of AKT including FoxO1a, PRAS40, FoxO3a, Tsc2, eIF4B, GSK-3 α , S6-Ser235, S6-Ser236 and mTOR, but some were previously unknown such as MAP4K4, ATRX, UCK2 and Bcl10 as well as those implicated in RNA splicing, e.g. WFS1 & ISWI (Figure 1.16A). Whilst the phosphoproteomes of AKT showed isoforms sharing much identical or at least some overlapping substrate specificity, there were significant differences between the phosphoproteomes of each AKT isoform (Figure 1.16B), suggesting that their functional differences are due to

1.3.3.4 Crystal structures of AKT

The crystal structures of the AKT2 kinase domain have been determined (Yang et al., 2002a, Yang et al., 2002b, Huang et al., 2003) as well as crystal structures of the AKT1 PH domain bound to PIP₃ (Thomas et al., 2002, Milburn et al., 2003) (Figure 1.17). These crystal structures have proved useful in the creation of drugs which target these regions. AKT inhibitors can target either the PH domain, preventing the recruitment of AKT to the lipid membrane, or the kinase domain, where AKT is still recruited to the membrane but cannot phosphorylate downstream substrates.

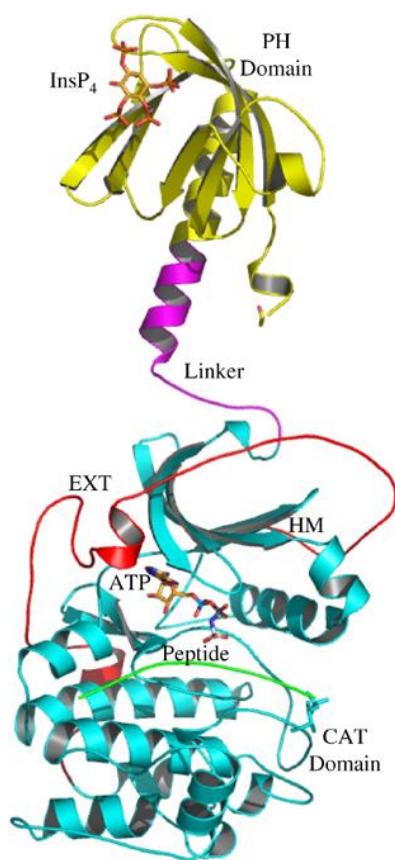


Figure 1.17: AKT crystal structure

Composite of crystal structures of the AKT1 PH (1UNQ) and AKT2 kinase (1O6K) domains. The PH domain was positioned arbitrarily relative to the kinase domain and a connector was modeled. Also a segment was added to connect two fragments of EXT. Segments are color coded: PH (yellow), InsP₄ (orange, red), LINK (magenta), CAT (cyan), EXT (red), AMP-PNP (atom colors), peptide substrate (green). Figure constructed by Jose Duca using PyMOL (DeLano, WL The PyMOL Molecular Graphics System (2002); DeLano Scientific, San Carlos, CA, USA. <http://www.pymol.org>). Figure and legend taken from a review (Kumar and Madison, 2005).

1.3.3.5 AKT interacting proteins and their functions

AKT is known to interact with many other proteins, including itself, forming oligomers (Datta et al., 1995). AKT interacting proteins can be grouped according to

the domains of AKT where they interact (Figure 1.18) (Du and Tsichlis, 2005).

TCL1, JIP1, GRB10 and RasGAP all bind to AKT at the N-terminus.

TCL1 (T-cell leukemia/lymphoma protein 1A) is a 14kDa protein consisting of 114 amino acids. It binds to the PH domain of AKT, increasing AKT kinase activity, leading to enhanced survival and proliferation (Laine et al., 2000). Whether binding of TCL1 to AKT facilitates AKT phosphorylation at Ser 473 or Thr 308 is debatable (Laine et al., 2000, Pekarsky et al., 2000). TCL1 increases AKT kinase activity up to ten-fold and its binding to AKT promotes AKT nuclear translocation (Pekarsky et al., 2000). However, the formation of TCL1/AKT complex is mediated at the plasma membrane by an exposed hydrophobic domain on TCL1 and the TCL1/AKT1 complex is generally concentrated in the plasma membrane and cytoplasm of B cells (French et al., 2002). TCL1 forms trimers which associate with AKT, facilitating AKT oligomerisation and activation (Noguchi et al., 2007).

JNK interacting protein 1 (JIP1) can bind AKT1 and make AKT1 unavailable for the assembly of JNK activation complexes. Growth factor receptor binding protein – 10 (GRB10) interacts with AKT via the PH domains. Since GRB10 binds to receptors via SH2 domains, GRB10 is likely to contribute to AKT activation by growth factors. Ras GTPase-activating protein (RasGAP) interacts with the PH domain of AKT and promotes AKT activation in response to serum stimulation (Du and Tsichlis, 2005).

There are also proteins that interact with AKT at the kinase domain. HSP90/CDC37 seems to prevent AKT from degradation by binding to the kinase domain of AKT. TRB3, binds the kinase domain in the activation loop and inhibits its phosphorylation at both Thr308 and Ser473 by growth factors and insulin. Adaptor protein containing PH domain, PTB domain and Leucine zipper motif (APPL) also binds to kinase domain, but the significance of this interaction in the regulation of AKT is not known (Du and Tsichlis, 2005).

C-terminal modulator protein (CTMP), AKT phosphorylation enhancer (APE), ArgBP2 γ , prohibitin 2 (PHB2) and PDK1 all interact with the C-terminal tail of AKT. CTMP inhibits the kinase activity of AKT, whereas APE enhances basal phosphorylation of AKT and seems to prolong the PI3K-dependent phosphorylation. Binding of Arg-binding protein 2 γ (ArgBP2 γ) to AKT does not seem to affect kinase

activity; it may contribute to the regulation of p21-activated protein kinase (PAK1) by AKT. The binding of AKT to PHB2 relieves the prohibitin-mediated repression of chromatin-associated transcriptional regulators. Finally, PDK1 interacts with AKT to facilitate threonine 308 phosphorylation.

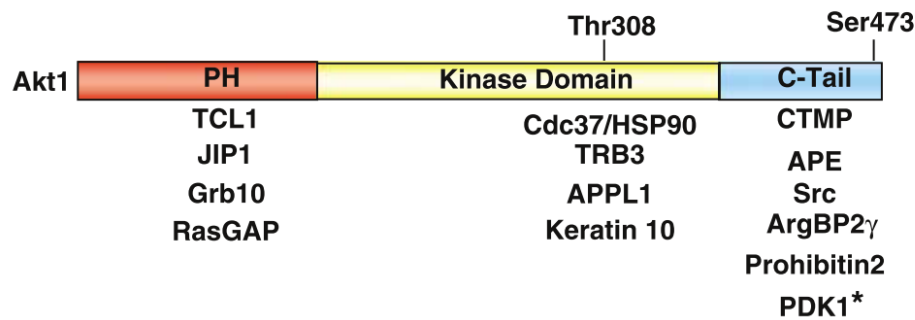


Figure 1.18: Interacting proteins bind specifically to different domains of AKT, resulting in either promoting or inhibiting AKT activation.

Diagram illustrating the domain architecture of the AKT protein. AKT interacting partners are listed under the domain they interact with. *The interaction of PDK1 with AKT at C-terminal is supported by a host of experimental data, but it has not been formally demonstrated. Figure taken from a review (Du and Tsichlis, 2005).

1.3.4 Signalling downstream of AKT

AKT is a central node in the PI3K signalling pathway. There are many downstream effectors of AKT, their phosphorylation by AKT resulting in many differing cellular functions (Figure 1.19).

The evidence regarding whether a specific downstream substrate is a real AKT substrate comes from pharmacological, genetic deletion and gain of function studies. Also, using algorithm motif-profiling to search for AKT substrates, FKHR, 6-PF2-K, GSK3 β , GSK3 α and mTOR were identified as the most stringent hits (Lawlor and Alessi, 2001, Yaffe et al., 2001). Many of the original studies regarding downstream signalling of AKT did not examine which isoform of AKT was involved. I will describe the consequences of these downstream substrates after phosphorylation by AKT in the following categories: inhibition of apoptosis and cell survival, cell cycle progression/proliferation, cell growth via mTOR and metabolism. It is important to note that these categories are arranged arbitrarily as the function of these phosphorylated substrates is likely to be overlapping between the categories (Manning and Cantley, 2007).

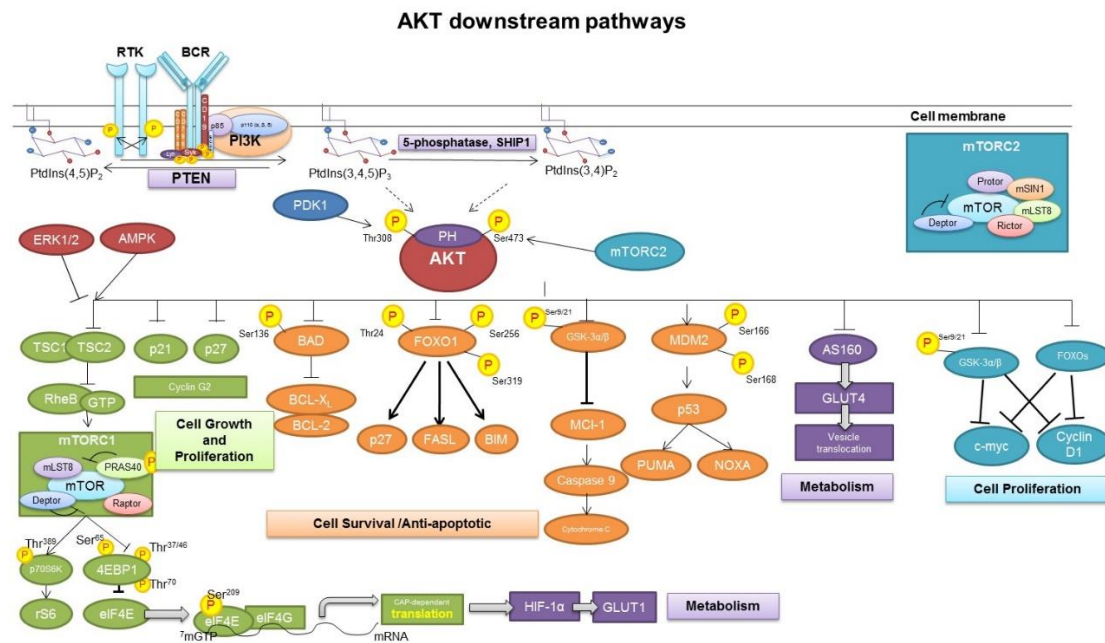


Figure 1.19: Class I PI3Ks activate AKT

Receptor-tyrosine kinase (RTK) and B Cell Receptor stimulated class I phosphoinositide 3-kinases, (removing the inhibitory action of the p85 regulatory subunit on the catalytic p110 subunit) (PI3Ks) generate phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃) and, via 5'phosphatase, SHIP, PtdIns(3,4)P₂. PIP₃ allows for the recruitment of the pleckstrin homology (PH) domain of AKT, driving translocation of AKT to the plasma membrane. AKT is phosphorylated on Ser473 by mammalian target of rapamycin complex 2 (mTORC2), followed by its phosphorylation on Thr308 by phosphoinositide-dependent kinase 1 (PDK1). Once fully active, AKT drives phosphorylation of a plethora of downstream protein targets, a selection of which is shown. These ultimately regulate cell growth and proliferation, cell survival, metabolism and proliferation. Figure based on a review (Vanhaesebroeck et al., 2012).

1.3.4.1 Inhibition of apoptosis and cell survival promotion

AKT phosphorylates many proteins including BAD, FOXOs, GSK3β and MDM2 leading to cell survival.

1.3.4.1.1 BAD

AKT regulates the interactions between the BCL-2 family of proteins. AKT-mediated phosphorylation of the pro-apoptotic BH3-only protein BAD on serine 136 provides a docking site for the adapter 14-3-3 to bind, which subsequently prevents BAD from interacting with anti-apoptotic BCL-2 family proteins, resulting in its loss of apoptotic function. As a result, this leads to an enhanced cell survival (Datta et al., 1997).

1.3.4.1.2 Caspase-9 and XIAP

AKT prevents apoptosis by directly phosphorylating and inactivating caspase-9. Caspases are intracellular proteases that function as effectors of apoptosis. AKT phosphorylates caspase-9 on serine196 and inhibits its protease activity, thus inhibiting apoptosis (Cardone et al., 1998). AKT1 and AKT2 interact with and phosphorylate X-linked inhibitor of apoptosis protein (XIAP) at residue serine 87. Phosphorylation of XIAP by AKT protects XIAP from either auto-ubiquitination or drug-induced ubiquitination and degradation (Dan et al., 2004). XIAP then inhibits activity of caspases, leading to resistance to apoptosis.

1.3.4.1.3 Forkhead (FOXO) transcription factors

The forkhead box class O (FOXO) family represents important transcription factors that are regulated by AKT. The FOXO family members include: FOXO1, FOXO3a, FOXO4 and FOXO6. FOXO1, FOXO3a and FOXO4 contain conserved serine/threonine residues (threonine 24, serine 256, and serine 319) that can be phosphorylated by AKT.

Phosphorylation of FOXOs causes FOXOs to relocate from the nucleus to the cytoplasm in a 14-3-3-dependent manner, where they are later degraded. As FOXOs are required for expression of genes that are involved in cell cycle arrest (e.g. p27) (Medema et al., 2000) and the induction of apoptosis (e.g. BIM and FAS ligand) (Kops and Burgering, 1999, Khwaja, 1999), phosphorylation of FOXOs by AKT results in enhanced cell proliferation and resistance to apoptosis.

1.3.4.1.4 GSK3

GSK3 was the first physiologically relevant substrate of AKT to be identified. AKT was shown to phosphorylate both GSK3 α and GSK3 β on serine residues in the amino terminus (serine 21 in GSK3 α and serine 9 in GSK3 β), resulting in inhibition of function (Cross et al., 1995). Using an algorithm motif-profiling search for AKT substrates, it predicts that AKT phosphorylates both GSK3 α and GSK3 β , but that it might have a higher affinity for GSK3 β (Lawlor and Alessi, 2001, Yaffe et al., 2001).

There are many established substrates of GSK3 α and GSK3 β , including glycogen synthase, eIF2B, ATP citrate lyase, Axin, β -catenin, APC, MUC1/DF3, cyclin D1, Jun, Myc, NFATc, C/EBPa, C/EBPb, CREB, MITF, HSF-1, Tau, MAP1B and presenilin-1. These proteins are involved in glycogen, protein and fatty acid synthesis, Wnt signalling, cell division, transcription and cytoskeleton changes (Frame and Cohen, 2001). GSK3 is therefore at the cross-roads between cell death and cell survival (Maurer et al., 2014). Here I shall discuss the functions of a few well-characterised substrates of GSK3 α and GSK3 β , such as glycogen synthase, cyclin D1, c-Myc, and MCL1 (Frame and Cohen, 2001).

Phosphorylation of GSK3 by AKT inhibits the activity of GSK3 and causes its substrates glycogen synthase (the enzyme that catalyses the last step in glycogen synthesis) and the eukaryotic initiation factor 2B (eIF2B) to become dephosphorylated by protein phosphatases (and thereby activated), promoting glycogen synthesis and protein synthesis respectively (Cohen and Frame, 2001).

GSK3 phosphorylates cyclin D1 at threonine 286 and the transcription factor c-Myc at threonine 58, resulting in translocation of these proteins from the nucleus to the cytoplasm where they are targeted for ubiquitylation and subsequent proteolytic destruction. Cyclin D1 binds to CDK4 and CDK6 and activates both kinases, thus facilitating entry into S-phase of the cell cycle. When cyclin D1 is degraded, this step of the cell cycle is inhibited. Phosphorylation of GSK3 by AKT results in reduced activity of GSK3 and increased expression of cyclin D1, thus promoting cell cycle progression. Phosphorylation of GSK3 also enhances the ability of c-Myc to stimulate cell proliferation and survival.

MCL1 an anti-apoptotic protein that is phosphorylated by GSK3 at a conserved site (serine159), leading to its degradation. Thus, phosphorylation of GSK3 by AKT leads to increased MCL1 stability and survival.

It is worth mentioning that AKT is not the only kinase known to phosphorylate and inactivate GSK3. Cyclic-AMP-dependent protein kinase/protein kinase A (PKA) in response to agonists that elevate the intracellular concentration of cAMP and MAPK-activated protein kinase-1 (MAPKAP-K1, also called RSK) or p70 S6 kinase (Sutherland and Cohen, 1994) can also catalyse the phosphorylation of GSK3 (Cohen and Frame, 2001).

1.3.4.1.5 MDM2

AKT has been shown to phosphorylate Mouse double minute 2 homolog (MDM2) also known as E3 ubiquitin-protein ligase MDM2 at serine 166 and serine 168, leading to its increased nuclear translocation (Mayo and Donner, 2001). MDM2 is an E3 ubiquitin ligase specific for p53. Accumulation of MDM2 in the nucleus promotes p53 degradation, thus resulting in a decrease in p53-mediated induction of apoptosis and enhanced cell survival.

1.3.4.1.6 AKT and the NFκB pathway

AKT is known to activate the NFκB signalling pathway after stimulation with TNFα or PDGF (Romashkova and Makarov, 1999, Ozes et al., 1999). AKT phosphorylates IκB kinase α (IKKα) at threonine 23, resulting in its degradation via the proteasome (Ozes et al., 1999). IKKα normally inhibits activation of NFκB and its loss facilitates the activation of NFκB, therefore increasing the transcription of NFκB target genes, such as BCL-XL and A1, promoting survival (Kane et al., 1999, Madrid et al., 2000, Beraud et al., 1999).

1.3.4.2 Cell cycle progression/proliferation

There are three members of the Kip family of cyclin dependent kinase inhibitors (CDKis) p21, p27 and p57, which inhibit corresponding CDKs to prevent the progression from the G₁ to S phase of the cell cycle.

AKT negatively regulates CDKis to facilitate cell cycle entry at two levels. Firstly, AKT phosphorylates FOXOs, as previously mentioned, and consequently inhibits their transcription of CDKis such as p27. Secondly, AKT has been shown to phosphorylate all three CDKis, p21 at threonine145 (Zhou et al., 2001a), p27 at threonines 157 and 198 (Shin et al., 2002, Liang et al., 2002) and p57 at serine 282 and threonine 310 (Lo, 2013, Zhao et al., 2013). As a result, phosphorylated CDKis were exported out of the nucleus into the cytoplasm where they are ubiquitinated and degraded via the proteasome. When the CDKis are down-regulated, the CDKs are liberated, which then drives cycle cell progression through G₁ phase, promoting cell proliferation.

1.3.4.3 Cell growth, protein synthesis and proliferation

1.3.4.3.1 mTOR

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase. It contains 20 or more HEAT repeats at the N-terminus, followed by a FAT domain (which modulates catalytic activity), an FRB domain which facilitates binding of FKBP-12, and the kinase domain at the C-terminus (Jacinto and Hall, 2003).

mTOR resides within the cell as two distinct multi-protein complexes, mTORC1 and mTORC2, which are distinguished by their distinct binding partners, their substrate specificities and their sensitivity to rapamycin. mTORC1 is usually referred to as a complex of mTOR and raptor and is sensitive to rapamycin, although it also contains mLTS8, PRAS40 and deptor. PRAS40 is a negative regulator of mTOR as it prevents the interaction between mTOR and its substrates. Deptor negatively regulates mTOR. mLTS8 lacks kinase activity but is required for mTORC1 to function correctly. mTORC2 comprises mLTS8 and deptor, mSIN1 and protor-1 and is rapamycin insensitive (Chapuis et al., 2010).

AKT activates mTOR complex 1 (mTORC1) in two ways, firstly by directly phosphorylating PRAS40 and secondly by indirectly phosphorylating the TSC1-TSC2 complex. When PRAS40 is phosphorylated by AKT, it dissociates from mTORC1 and releases mTORC1 for activity. Multisite phosphorylation of TSC2 within the TSC1-TSC2 complex blocks the ability of TSC2 to act as a GTPase-activating protein (GAP) for Rheb, thereby allowing Rheb-GTP to accumulate which then activates mTORC1.

Energy sensing of the cell is performed by LKB1 and AMPK. LKB1 mediates activation of AMP-activated protein kinase (AMPK) and AMPK inhibits mTOR. AMPK senses the AMP/ATP (adenosine triphosphate) ratio and is therefore considered to be the cell's 'energy sensor'. Under energy stress, (high AMP/ATP), AMPK phosphorylates TSC2 on T1227 and S1345 which leads to repression of mTORC1 (Inoki et al., 2003).

The amino acids leucine and arginine are the principle stimulators of mTORC1. RAG1 and RAG2 proteins are small GTPases of the Ras superfamily. RAGs can

bind mTORC1 and relocate it to late endosomal compartments which contain Rheb-GTP activators (Kim et al., 2008, Sancak et al., 2008).

A cell senses hypoxia via 'regulated in development DNA damage responses 1' (REDD1) (via TSC2) and controls amino acid intake.

mTOR signals downstream to a minimum of two independent targets to control mammalian cell growth. S6K1 directly phosphorylates ribosomal protein S6, which enhances translation of transcripts with 5'terminal oligopyrimidine (Jefferies et al., 1997). Other targets of S6K1 include CBP80 (RNA exporting/splicing), CREM- τ - (Transcription), eEF2- (Translation), and BAD (Cell survival). Phosphorylation of 4EBP1, allows eIF4E to dissociate and assemble with eIF4G to facilitate cap-dependent translation (Gingras et al., 2001).

mTOR is therefore considered the "hub" of cell growth; it integrates growth factor signalling, usually via AKT, energy levels (via AMPK via TSC2), nutrient intake (amino acids) and downstream processes (Figure 1.20).

AKT and mTOR signalling controls cell size

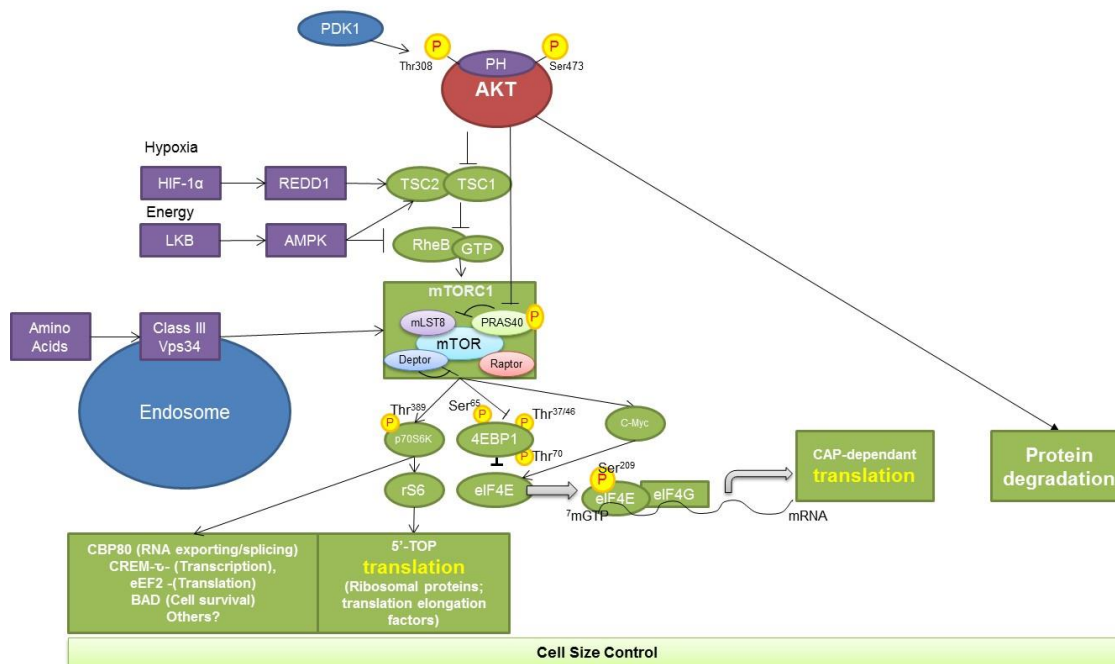


Figure 1.20: mTOR signalling controls cell size.

mTOR dependent and mTOR independent pathways of AKT mediated increases in cell size. mTOR can thus be considered the 'hub' that regulates cell growth, that regulates extracellular signals, nutrient intake and downstream processes. AKT is an important activator of mTOR. mTOR senses energy levels via AMPK (via TSC2), senses hypoxia via REDD1 (via TSC2) and controls amino acid intake. mTOR signals downstream to a minimum of two independent targets to control mammalian cell growth. S6K1 directly phosphorylates ribosomal protein S6, which enhances translation of transcripts with 5' terminal oligopyrimidine (Jefferies et al., 1997). Other targets of S6K1 include CBP80 (RNA exporting/splicing), CREM- τ (Transcription), eEF2 (Translation), BAD (Cell survival). Phosphorylation of 4EBP1, allows eIF4E to dissociate and assemble with eIF4G to facilitate cap-dependent translation (Gingras et al., 2001). AKT also directly regulates protein degradation (Faridi et al., 2003). Diagram and legend based on figures from (Faridi et al., 2003, Fingar et al., 2002, Chapuis et al., 2010).

1.3.4.3.2 AKT, mTOR and cell size.

AKT affects cell size. Genetic deletion of AKT resulted in reduced cell size in *Drosophila* (Verdu et al., 1999) and conversely constitutively active AKT1 in transgenic mice increased cell size (Shioi et al., 2002). In an animal study, AKT has been shown to promote muscle hypertrophy by activating the mTOR pathway and inhibiting GSK3 activity (Rommel et al., 2001). In the breast cancer MCF-7 cell line, cells that express constitutively active AKT1 or AKT3 have increased cell size. Activation of AKT1 in another cell line caused a two-fold increase in cell size (Faridi et al., 2003).

AKT promotes increase in cell size via mTOR by stimulating protein synthesis and inhibiting protein degradation (Faridi et al., 2003) as well as increasing nutrient

uptake (Edinger and Thompson, 2002). mTOR signals downstream via S6K and eIF4E (two independent targets) to control mammalian cell growth (Fingar et al., 2002).

Cell growth is generally considered to be essential for cell division. In order for a cell to divide, it must not be either too large or too small, otherwise the mitotic spindle will struggle to form correctly, due to the physical properties of macromolecules such as microtubule and chromosomes (Marshall et al., 2012). Cell growth and cell cycle progression are, however, separable processes in mammalian cells, though exactly how cell growth and cell cycle are co-ordinated is still incompletely understood (Fingar et al., 2002). Historically, it was thought that cell cycle progression is dependent on cell growth, leading to the speculation of a cell growth checkpoint (Johnston et al., 1977). However, mammalian cells were able to proliferate with reduced size, indicating that, if a growth checkpoint exists, it must be dynamic and responsive to the extracellular milieu (Fingar et al., 2002).

It is noteworthy that, whilst inhibition of mTOR by rapamycin decreased cell size in all phases of the cell cycle, this was most noticeable in G1 phase, suggesting AKT-mTOR dependent cell growth primarily occurs in G1 (Fingar et al., 2002). However, using a cell line expressing a constitutively active AKT3, it was shown that cell size increase of 20% has been observed in all stages of the cell cycle (Faridi et al., 2003).

Finally, rapamycin only inhibited 70% of AKT-induced increase in cell size (Faridi et al., 2003), suggesting that, although AKT mediates most of the effect on cell size via mTOR, AKT may also regulate cell size via other mechanisms. For instance, AKT phosphorylates and inactivates GSK3, which is an inhibitor of eIF2B as well as 4EBP1 (Gingras et al., 1998). Another mechanism by which AKT regulates cell size is inhibiting protein degradation in an mTOR-independent manner (Faridi et al., 2003) (Figure 1.20).

1.3.4.4 Glucose metabolism

AKT is involved in glucose metabolism in several ways. AKT2, in particular, is involved in transportation of the GLUT4 glucose transporter to the plasma membrane via RabGAP AS160 (Gonzalez and McGraw, 2009).

AKT phosphorylates 6-phosphofructo-2-kinase (PFK2), an enzyme involved in glycolysis (Deprez et al., 1997). AKT promotes glycolysis and maintains a physiologic mitochondrial membrane potential (Plas et al., 2001). AKT increases coupling of glucose metabolism to oxidative phosphorylation (Gottlob et al., 2001).

AKT has long been an attractive drug target due to its involvement in regulating many cellular pathways. However since one of those pathways is involved in insulin signalling and glucose regulation, targeting AKT results in an ‘on target’ side effect of hyperglycaemia (Banerji, 2013). A combination of fasting and low carbohydrate diet has been used to reduce the magnitude of hyperglycemia induced by an AKT inhibitor in mice (Crouthamel et al., 2009).

1.3.4.5 NO production

AKT can phosphorylate endothelial nitric oxide (NO) synthase (eNOS) on serine 1177, leading to its activation. The release of NO that is produced by eNOS results in vasodilation, vascular remodelling and angiogenesis (Dimmeler et al., 1999, Fulton et al., 1999).

1.3.4.6 RNA splicing

AKT was recently shown to be involved in RNA splicing. IWS1, an RNA processing regulator, is phosphorylated by AKT3 and AKT1 at serine 720 and threonine 721, and in mice model of lung cancer, phosphorylated IWS1 has been shown to contribute to tumour growth and invasiveness (Sanidas et al., 2014, Lee and Blenis, 2014).

1.3.4.7 Inhibition of autophagy

Autophagy is the process in which a portion of the cytoplasm is wrapped into a specific membrane-bound vesicle, which is then fused with lysosome and its contents digested by lysosomal enzymes. Autophagy is believed to serve as a cytoprotective mechanism in extreme conditions such as starvation, or as an alternative pathway of cell death, especially when apoptosis is inhibited (Kroemer and Jaattela, 2005). AKT suppresses autophagy. AKT phosphorylates beclin 1, enabling its interactions with 14-3-3 and vimentin intermediate filament proteins to form an autophagy-inhibitory complex (Wang et al., 2012b). Cells expressing a constitutively active AKT display

suppression of autophagy induced by rapamycin, whereas dominant negative AKT promoted autophagy induced by rapamycin or LY294002 (Noguchi et al., 2014). Therefore inhibition of AKT can enhance autophagy.

1.4 PI3K and AKT in CLL

1.4.1 PI3K in CLL

Although gain-of-function mutations in *PIK3CA* gene (coding for PI3K p110 α) are commonly reported in many solid tumours (Samuels et al., 2004, Stemke-Hale et al., 2008), there is a very infrequent occurrence of *PIK3CA* mutations in CLL, with such mutation detected in only one out of sixty-one samples (Marincevic et al., 2009). Another study showed no point mutations in *PIK3CA*, *PIK3CD*, *PIK3CG*, *PTEN* and *PIK3RI* and *AKTE17K* in 188 CLL samples examined, suggesting that point mutations are not a major mechanism of activation of the PI3K pathway in CLL (Brown et al., 2012).

However, copy number analysis showed that 5.6% of patients (9 of 161) had 3q26 amplifications where *PIK3CA* gene is located. The amplification of *PIK3CA* was shown to associate with poor prognostic factors, such as ZAP70 positivity, CD38 presence and higher total number of treatments (Brown et al., 2012).

Meanwhile, it has also been shown that PI3K mRNA was significantly overexpressed in UM-CLL compared to M-CLL (1.7 fold increase) (Kienle et al., 2006). PI3K expression at protein level was also examined in 185 CLL samples by immunohistochemistry. Winkler and colleagues reported that protein levels of PI3K were 1.5-fold higher ($p=0.01$) in the subgroup with normal karyotype (Winkler et al., 2010). However, no comparison in expression of PI3K was made between UM-CLL and M-CLL samples.

1.4.1.1 Expression of p110 isoforms in CLL

PI3K activity is higher in CLL cells than normal B cells and PI3K p110 δ isoform is abundantly expressed in CLL cells (Herman et al., 2010). In another study, it was reported that primary CLL cells express abundant p110 δ and p110 β , modest amounts of p110 γ and undetectable levels of p110 α (Balakrishnan et al., 2015). However, the significance of these finding is overshadowed by the small number of CLL samples

used in the latter study (n=3). Therefore, other than p110 δ , the expression of class I PI3K p110 isoforms has not been extensively examined in CLL cells.

1.4.1.2 Is PI3K constitutively active in CLL?

PI3K is important for CLL cell survival, since inhibiting PI3K with a pan-PI3K inhibitor, LY294002, led to apoptosis (Bernal et al., 2001, Ringshausen et al., 2002). However, whilst confirming that AKT was present in CLL cells, Ringshausen and colleagues found little or no phosphorylated AKT and suggested that PI3K mediated its survival activity via protein kinase C δ (PKC δ) (Ringshausen et al., 2002). Other papers have nevertheless suggested that PI3K is constitutively active in CLL cells, based on observations that phosphorylated AKT was detected in unstimulated CLL cells (Plate, 2004, Zhuang et al., 2010, de Frias et al., 2009, Barragan et al., 2006). PI3K is also required to mediate the pro-survival signalling following activation of the BCR or CD40 in CLL cells as PI3K inhibitors reduced survival in stimulated cells (Bernal et al., 2001, Cuni et al., 2004). Therefore, accumulating evidence suggests that PI3K is active in CLL cells and plays an important role in CLL-cell survival.

1.4.1.3 PI3K in CLL cell survival

The PI3K p110 δ -selective inhibitor idelalisib induced apoptosis in a dose- and time-dependent fashion in primary CLL cells, regardless of their *IGHV* mutational status or whether or not they carried cytogenetic abnormalities. Idelalisib induced more apoptosis in CLL cells than B cells and did not cause cytotoxicity in T or NK cells, implicating a pivotal role of PI3K p110 δ in the survival of CLL cells (Herman et al., 2010). Inhibition of all PI3K isoforms (using LY294002) also induced apoptosis, but indiscriminately across all cell types, thus suggesting that PI3K δ is particularly important in the survival of CLL cells and not of other haematopoietic cells. Inhibiting PI3K p110 δ and p110 γ using IPI-145 (also known as duvelisib) also induced apoptosis in CLL cells independently of prognostic markers. IPI-145 also induced apoptosis in CLL cells stimulated with BCR cross-linking using anti-IgM or co-cultured with stromal cells, thus implicating PI3K as important for CLL cell survival (Balakrishnan et al., 2015). BKM210, a pan class I PI3K inhibitor, was also able to induce apoptosis of CLL cells *in vitro*, even in the presence of stromal cell support (Amrein et al., 2013). Recently it was shown that CXCR4^{hi} CD49d^{hi} mutated

IGHV CLL cases are more susceptible to the apoptotic effect of PI3K δ inhibition than CXCR4^{low} CD49d^{low} mutated *IGHV* CLL, suggesting that CXCR4^{hi} CD49d^{hi} mutated *IGHV* CLL cases are more dependent on PI3K δ for survival (Pepper et al., 2015).

Copanlisib (BAY 80-6946), a PI3K- α/δ inhibitor was more cytotoxic to CLL cells than either idelalisib (15-fold more cytotoxic) or duvelisib, suggesting that PI3K p110 α may also have a role in CLL-cell survival (Gockeritz et al., 2015).

PI3K p110 α (rather than PI3K- δ or PI3K- β) was also shown to be important in mediating the survival effects of stromal cells on CLL cells as p110 α inhibitors enhanced the cytotoxicity of fludarabine and reversed the protective effect of stromal cells on fludarabine-induced apoptosis (Niedermeier et al., 2009).

It has also been shown that inhibition of p110 α , β and δ (using isoform selective class I PI3K inhibitors) induced apoptosis whereas inhibition of p110 γ did not affect the viability of CLL cells, suggesting that p110 α , β and δ are contributing to survival of CLL cells (de Frias et al., 2010).

Therefore, based on the published literature to date, it is conceivable that PI3K p110 α , β and δ are all contributing to CLL-cell survival, with PI3K p110 δ being the major contributor.

1.4.1.4 Role of PI3K in CLL microenvironment

As described in the previous section ([1.1.4.2](#)), CLL is essentially a microenvironment-dependent disease. The role of PI3K in mediating signalling between CLL and its microenvironment has been unravelled, largely owing to the fact that the isoform selective inhibitors of PI3K were made available and used in these studies.

Below, I shall summarise the published data demonstrating a specific role of PI3K in regulating some important biological activities of CLL cells in the tissue microenvironment, namely survival, migration, adhesion and proliferation.

1.4.1.4.1 CLL-cell survival

The PI3K p110 δ inhibitor, idelalisib, at a low concentration (0.1 μ M, which also prevents phosphorylation of AKT at serine 473 upon CD40 stimulation) has been shown to inhibit T cells from producing cytokines IL-6, IL-10 and TNF- α , as well as CD40L mRNA (Herman et al., 2010). Idelalisib also inhibited NK cells from producing IFN- γ . These cytokines are all involved in promoting proliferation and survival of CLL cells in the tissue microenvironment (Ghia et al., 2008, Burger, 2011). In addition, idelalisib effectively blocked the protective effects on CLL cells stimulated with BAFF, TNF α and through direct contact with stromal cells or fibronectin adhesion, implicating a role of PI3K p110 δ in mediating pro-survival signalling from microenvironment stimuli (Herman et al., 2010).

PI3K p110 δ also mediates survival signals initiated from BCR-activation or derived from NLCs in CLL cells. Co-culturing CLL cells with NLCs results in increased CCL2, CCL7, IL-6, sCD154, CCL22, TNF α and CCL17 secretion and the addition of idelalisib reduced the secretion of all of these chemokines and cytokines (Hoellenriegel et al., 2011). Activation of CLL cells by co-culture with NLCs and via direction stimulation of the BCR using anti-IgM results in increased secretion of CCL3 and CCL4 (Burger et al., 2009); both idelalisib and IPI-145 significantly inhibit their secretion (Hoellenriegel et al., 2011, Balakrishnan et al., 2015).

1.4.1.4.2 CLL-cell migration

The ability of CLL cells to migrate towards CXCL12 and CXCL13 is dependent upon p110 δ (Hoellenriegel et al., 2011). Both idelalisib and PI3K p110 δ and p110 γ dual inhibitor IPI-145 inhibited the chemotaxis towards CXCL12 and CXCL13 (Balakrishnan et al., 2015). Recently it was shown that idelalisib significantly reduced expression of CXCR4 and CD49d (both molecules important in CLL migration). Idelalisib also induced a greater percentage reduction in the migration of CXCR4^{hi} CD49d^{hi} mutated *IGVH* CLL cases compared to CXCR4^{low} CD49d^{low} mutated *IGHV* CLL (Pepper et al., 2015). Further, BCR signalling suppresses S1PR1 expression via a mechanism involving PI3K p110 δ , thereby inhibiting egress from the lymph node environment (Till et al., 2015).

Copanlisib (BAY 80-6946), a PI3K- α/δ dual inhibitor, inhibited migration more than either idelalisib, or duvelisib, suggesting that PI3K p110 α may also be involved in CLL cell migration (Gockeritz et al., 2015).

CXCL12 is secreted by marrow stromal cells (MSC) and attracts CLL cells via its cognate receptor CXCR4. CXCR4 is essential for homing and retention of CLL cells in the bone marrow microenvironment and activation of CXCR4 by CXCL12 protects CLL cells from spontaneous and drug-induced cell death (Burger and Burkle, 2007). PI3K- α (rather than PI3K- δ or PI3K- β) was shown to be important in mediating the effects of chemotaxis and actin polymerisation in response to CXCL12 and migration beneath MSC (Niedermeier et al., 2009). Therefore, based on the published literature so far, PI3K α and PI3K δ appear to be involved in regulating CLL migration.

1.4.1.4.3 CLL-cell adhesion

Adhesion of CLL cells to stromal cells in bone marrow requires interactions between VLA-4 integrin ($\alpha 4\beta 1$, also known as CD49d/CD29) expressed on CLL cells and VCAM-1 and fibronectin on stromal cells, which is further strengthened through activation of CLL cells via the CXCR4 receptors by the cognate ligand CXCL12 that is abundantly expressed by MSCs (Burger and Kipps, 2002). It has been shown that this adhesion is mediated by PI3K p110 δ as idelalisib inhibited integrin-mediated adhesion of CLL cells to marrow stromal cells (Fiorcari et al., 2013).

In another study, it was shown that adhesion of CLL cells to stromal cells can be enhanced by IL-8, IL-6 or CD40L + IL-4. Stimulatory effects of IL-8 or IL-6 on adhesion are fully inhibited by a potent pan-PI3K inhibitor, PI-103, while the effects of CD40L + IL-4 are only partially reversed (Lafarge et al., 2014). Therefore the enhanced adhesion by IL-8 or IL-6 is also mediated by PI3K.

1.4.1.4.4 CLL-cell proliferation

As described in section [1.1.4.3.5](#) CLL cells proliferate in the lymph node not the bone marrow (van Gent et al., 2008, Herishanu et al., 2011, Pascutti et al., 2013). One important mechanism responsible for induction of proliferation involves interaction between T cells and CLL cells in the lymph node microenvironment, most likely through activating the CD40L-CD40 signalling pathway (Os et al., 2013,

Pascutti et al., 2013, Ahearne et al., 2013). Thus it has been shown that CLL cells were induced to proliferate *in vitro* in response to stimulation by CD40L + IL-10+IL+2 and this proliferation was effectively inhibited by the PI3K p110 δ inhibitor, idelalisib and IPI-145, a dual PI3K p110 δ / γ inhibitor (Balakrishnan et al., 2015). This suggests that PI3K p110 δ , p110 γ , or both, are involved in mediating the proliferative signals of CD40L + IL-10 + IL+2.

Based on the above evidence, it is reasonable to conclude that PI3K p110 δ play a major role in mediating survival, migration, adhesion and proliferation of CLL cells in the tissue microenvironment. Other isoforms of PI3Ks such as p110 α and p110 γ may also contribute to CLL survival, migration and proliferation.

1.4.1.5 Targeting PI3K in CLL

Due to the importance of PI3K in mediating intrinsic and extrinsic survival signals in CLL, targeting PI3K has been considered as an important approach to improve the treatment of the disease. However, because the PI3K pathway is also critical to many essential physiological functions of normal cells and tissues such as metabolism, growth, proliferation and survival, pharmacological inhibition of PI3K on a global scale leads to many undesirable toxicities (Yap et al., 2015). The selective inhibition of PI3K p110 δ thus makes it an ideal therapeutic approach in CLL as it reduces the toxicity associated with pan-PI3K inhibitors by selectively targeting PI3K p110 δ -dependent cells such as CLL cells. Indeed, there have been significant advances over the last few years in developing isoform-specific inhibitors and below I shall describe some of the recent major developments.

1.4.1.5.1 Idelalisib

Since Idelalisib has been shown to have promising preclinical activity in CLL (Herman et al., 2010, Lannutti et al., 2011, Hoellenriegel et al., 2011), it has been evaluated in clinical studies. In an early phase clinical study, treatment with idelalisib as a monotherapy resulted in significantly improved responses and progression-free survival in patients with relapsed or refractory CLL with adverse prognostic features (Brown et al., 2014). Idelalisib treatment also led to decrease of AKT phosphorylation in CLL cells from patients and significantly reduced serum levels of CLL-related chemokines (e.g. CCL3, CCL4, and CXCL13). In another large,

multicentre phase III clinical study involving 220 unfit patients with relapsed CLL, idelalisib in combination with rituximab also achieved impressive clinical activity, significantly improving progression-free survival, response rate and overall survival among those patients (Furman et al., 2014b).

Idelalisib has been shown to enhance the killing by several cytotoxic agents of CLL cells co-cultured with marrow stromal cells *in vitro* (Hoellenriegel et al., 2011), increase the immunomodulatory effects of lenalidomide in CLL cells *in vitro* (Herman et al., 2011b) and synergise with histone deacetylase inhibitors in inhibiting proliferation and induction of apoptosis of CLL cells (Bodo et al., 2013), but the clinical activity of combining idelalisib with these agents remains to be seen.

Recently, the structural, biochemical, and biophysical characterization of binding of idelalisib to PI3K p110 δ was determined. The 2.4Å crystal structure of the inhibitor-PI3K p110 δ complex showed idelalisib binding in the ATP-binding site of the enzyme and revealed the specific and non-covalent interactions between the inhibitor and the protein (Somoza et al., 2015).

1.4.1.5.2 IPI-145 (duvelisib)

IPI-145 (duvelisib) is a dual PI3K δ/γ inhibitor, with IC₅₀ values for PI3K- δ and PI3K- γ of 2.5 nM and 27 nM, respectively (Winkler et al., 2013). Preclinical and early-phase clinical studies all demonstrated promising activity in CLL (Balakrishnan et al., 2015). Importantly, given the emergence of resistance to the BTK inhibitor ibrutinib in CLL, IPI-145 has been shown to be able to overcome ibrutinib-resistance in a model cell line mimicking C481S mutation in BTK (Dong et al., 2014).

1.4.1.5.3 Other PI3K inhibitors

Buparlisib (BKM120), a pan-class I PI3K inhibitor, was also able to induce apoptosis of CLL cells co-cultured with stromal cells (Amrein et al., 2013). The biological activity of BKM120 included the reduction in phosphorylation of AKT and FOXO3a, decrease in MCL1 expression and induction of BIM expression. BKM120 sensitised CLL cells to fludarabine and bendamustine and inhibited CLL cell migration to CXCL12 (Rosich et al., 2013).

Copanlisib (BAY 80-6946), a PI3K- α/δ inhibitor has also shown promising preclinical activity (Gockeritz et al., 2015). Copanlisib was more toxic to CLL cells than either idelalisib (15-fold more cytotoxic) or duvelisib (IPI-145) and more potently inhibited migration than either of these agents. Finally, Copanlisib was more toxic to CLL cells cultured on stromal support cells, than either idelalisib or duvelisib. Copanlisib lacked significant cytotoxicity against T cells. These results not only suggest that Copanlisib inhibitor might be useful in the clinic, but also that PI3K p110 α may have a significant role in CLL cell survival and migration (Gockeritz et al., 2015).

Pilaralisib (SAR245408/XL147), a pan-PI3K inhibitor, has been shown to be safe in phase I clinical study of CLL, with 50% of patients having a partial response. Pilaralisib significantly reduced plasma levels of several cytokines and chemokines involved in B-cell trafficking in CLL, namely: CXCL13, CCL3, CCL19, CCL22, TNFR2 and IL-2R α , and also reduced CCL4, although not significantly (Brown et al., 2015).

The dual PI3K/mTOR inhibitor PF-04691502 reduced CLL cell survival irrespective of cytogenetics or *IGVH* status. PF-04691502 inhibited both anti-IgM and stromal cell survival signals, as well as migratory stimuli from CXCL12 *in vitro*, and also reduced tumour burden in the E μ TCL1 mouse, suggesting this is a possible new PI3K therapy (Blunt et al., 2015).

1.4.1.6 Negative regulators of PI3K in CLL

PTEN (Phosphatase and tensin homolog) is an important negative regulator in the PI3K pathway, as it counteracts the action of PI3K by dephosphorylating PIP₃ to PIP₂, thereby controlling the activation of AKT. The role of PTEN in CLL is still not clear. In another leukaemia (T-ALL), PTEN action has been shown to be dependent on the microenvironment (Miething et al., 2014).

There are no *PTEN* gene mutations in CLL, however, the level of PTEN protein may play a role in CLL pathophysiology since PTEN protein expression was not detected in 11/41 CLL patient samples (28%) and was reduced in eight patients (20%) (Leupin et al., 2003). In contrast, a separate investigation studying phosphatase PHLPP1 in CLL detected PTEN protein expression in all CLL samples studied

(O'Hayre et al., 2012). It is therefore unclear what role, if any, PTEN may have in CLL.

PTEN mRNA expression was decreased in CLL compared to normal B cells. Decreased *PTEN* mRNA expression was shown to correlate with poor prognosis in CLL. Thus *PTEN* could be a novel prognostic indicator in individuals with CLL (Zou et al., 2013, Best and Mulligan, 2013).

Most recently, *PTEN* mRNA and protein were found to be down regulated in CLL cells co-cultured with CD40 +IL-4 (Palacios et al., 2014). There have been attempts at re-activating PTEN in CLL using CK2 inhibitors, leading to decreased PI3K-AKT signalling and therefore increased apoptosis (Shehata et al., 2010, Martins et al., 2010, Martins et al., 2014).

Finally, SHIP1 and SHIP2 are other phosphatases which hydrolyse PI(3,4,5)P₃ to generate PI(3,4)P₂ (Bunney and Katan, 2010). While SHIP2 protein was undetectable, SHIP1 protein was detected in primary CLL cells, but there is variation in expression of SHIP1 between CLL samples inversely associated with ZAP-70 expression (Gabelloni et al., 2007). The significance of this finding is still unclear.

1.4.2 AKT in CLL

1.4.2.1 Expression of AKT in CLL cells

As described earlier, there are three isoforms of AKT encoded by three separate genes in mammals. Despite the importance of AKT in mediating many important cellular functions, there is limited knowledge regarding the isoform-specific expression of AKT in CLL. Previous work has shown that AKT1 is expressed in CLL cells and plays a role in cell survival (Zhuang et al., 2010). Very recently, it was shown that AKT1, but not AKT2, is required for survival and chemoresistance of CLL cells, despite the expression of AKT2 being higher than AKT1 at both mRNA and proteins levels (Hofbauer et al., 2015). The expression of AKT3 in CLL cells has not been reported.

A transforming mutation in the PH domain of AKT1 (E17K) has been reported in some solid tumours in humans (Carpten et al., 2007, Bleeker et al., 2008). However,

the E17K mutation in AKT1 has not been detected in CLL or other B-cell lymphoid leukaemias (Mahmoud et al., 2008, Zenz et al., 2008a).

AKT interacting proteins have not been extensively studied in CLL, with the exception of TCL1. TCL1 is present in 90% of CLL cases and its overexpression correlates with poor prognostic indicators (Herling et al., 2006, Herling et al., 2009). TCL1-transgenic mice expand CD5⁺/IgM⁺ lymphocyte populations, which share some similarities with CLL in humans (Bichi et al., 2002), and lymphocytes from the transgenic mice have increased levels of phosphorylated AKT (Hoyer et al., 2002). This TCL-1 transgenic mouse has been suggested as a preclinical drug development tool for CLL (Johnson et al., 2006). TCL1 is critical for AKT activation in CLL cells; overexpressing TCL1 led to increased AKT activation, and conversely downregulating TCL1 led to reduced AKT activation (Hofbauer et al., 2010). AKT has also been shown to directly interact with TCL1A in CLL cells. Disrupting the AKT-TCL1 interaction may have therapeutic potential (Popal et al., 2010). In fact, peptide-based inhibitors that have been developed to interrupt protein interaction with TCL1 were potent in inhibiting AKT and in reducing CLL cell survival (Schrader et al., 2014).

1.4.2.2 AKT in CLL-cell survival

CLL cells normally undergo spontaneous apoptosis *in vitro*. Strangely, *AKT* gene expression was upregulated during culture of CLL cells (Plate, 2004). CLL-cell survival can be enhanced by culture conditions where chemokines, cytokines or accessory cells are present. Numerous studies have shown AKT to be important in the transduction of these survival signals. AKT was shown to be phosphorylated and to promote survival upon BCR stimulation, CXCL12, IL-4, TPA, CD40 and albumin stimulation.

In non-malignant B cells, BCR-induced activation of the PI3K-AKT-GSK3 β pathway promotes B cell survival, which is then dependent on LYN and SYK kinase for sustained AKT activation (Gold et al., 1999, Pogue et al., 2000, Donahue and Fruman, 2003). AKT is involved in the survival of CLL cells downstream of BCR. Bernal and colleagues were the first to show that signalling through BCR stimulation (using soluble anti-IgM antibody) promotes CLL-cell survival through PI3K-AKT and NF κ B pathways (Bernal et al., 2001). The PI3K inhibitor LY294002 inhibited

phosphorylation of AKT at Ser 473 and Thr308 and abolished the pro-survival effect after BCR stimulation. Later it was shown that BCR signalling, whether induced by soluble anti-IgM or immobilised anti-IgM antibody, resulted in AKT activation (Petlickovski et al., 2005). Stimulation of BCR by immobilised anti-IgM resulted in a longer prolonged phosphorylation of AKT and ERK, which led to efficient degradation of I κ B, the NF κ B inhibitor. Such BCR stimulation activated AKT in 100% of cases (irrespective of *IGVH* status) and caused no apoptosis. The inhibition of PI3K, using LY294002, abrogated the protective effect of BCR stimulation on chemotherapy-induced apoptosis (Petlickovski et al., 2005).

Longo and colleagues further showed that BCR stimulation of CLL cells using immobilised anti-IgM antibody results in AKT phosphorylation, and enhanced CLL cell survival (Longo et al., 2008). It was also shown that sustained AKT activation in CLL cells results in elevated expression of anti-apoptotic proteins including MCL1, BCL-XL and XIAP, whereas sustained ERK activation only results in elevated levels of XIAP. Knockdown of MCL1 resulted in a significant decrease in CLL cell viability, whereas knockdown of the other anti-apoptotic proteins did not have the same effect, thus suggesting that the AKT-MCL1 pathway is the primary cell survival pathway in CLL cells after BCR stimulation (Longo et al., 2008).

In agreement with Longo's work, Zhuang and colleagues also confirmed the role of the AKT-GSK3 α -MCL1 pathway in CLL-cell survival. Knockdown of AKT1 or pharmacological inhibition of AKT in CLL cells reduced levels of p-GSK3 α and MCL1 protein and induced CLL-cell apoptosis (Zhuang et al., 2010, de Frias et al., 2009).

It has also been shown that in the absence of BCR cross-linking GSK3 was phosphorylated, suggesting that AKT is constitutively active in CLL cells (Kawauchi et al., 2002).

CD40 stimulation (with anti-CD40 monoclonal antibodies) promotes CLL-cell survival through PI3K-AKT and NF κ B pathways (Bernal et al., 2001). CLL cells co-cultured with mouse fibroblasts alone survive longer by up regulating BCL-XL in a PI3K-dependent manner. AKT was phosphorylated at serine 473 in CLL cells upon co-culture with these mouse fibroblasts and this phosphorylation further increased by three-fold with the addition of anti-CD40 monoclonal antibodies (Cuni et al., 2004).

Similarly, the addition of soluble CD40 ligand to CLL culture medium for two hours led to the phosphorylation of AKT at serine 473 (Herman et al., 2010).

Albumin was shown to activate AKT in CLL and reduce apoptosis induced by chlorambucil and radiation, and the PI3K inhibitor LY294002 reversed this protective effect, suggesting that the PI3K-AKT pathway was involved in mediating albumin-induced survival signalling in CLL (Jones et al., 2003, Wickremasinghe et al., 2001).

AKT is activated in CLL cells upon co-culture with HS-5 stromal cells for five days, as measured by the ratio of p-AKT (Ser473) / total AKT (Hofbauer et al., 2010). Co-culture with bone marrow fibroblasts also induced AKT phosphorylation and enhanced survival of CLL cells (Edelmann et al., 2008).

Most of these aforementioned studies utilised the PI3K inhibitors LY294002 and wortmannin, or AKT inhibitors A-443654 and Akti1/2, which are now accepted to have off-target effects and there now exist more specific drugs inhibiting these kinases (Bain et al., 2007).

1.4.2.3 AKT in CLL-cell proliferation and cell cycle

Two papers by Longo and colleagues have highlighted the importance of AKT in CLL-cell proliferation (as measured by DNA synthesis) in response to CpG oligodinucleotides (CpG ODN) (Longo et al., 2007, Longo et al., 2008). CpG ODN mimics CpG motifs present in un-methylated bacterial DNA, which are recognised by toll-like receptor 9 (TLR9). The response of CLL cells to CpG-ODN is variable and cells from patients with progressive disease are more likely to proliferate. It was observed that CLL cells that proliferated had higher levels of phosphorylated AKT, ERK, JNK and p38 MAPK expression and that those that proliferated had about 6-fold more phosphorylated AKT than those that did not proliferate. It was also observed that proliferating CLL cells expressed cyclin E after 24 hours and cyclin A after 48 hours of CpG ODN stimulation, whereas the non-proliferating cells did not. Finally, Longo and colleagues showed that enforced expression of constitutively active AKT (myristoylated AKT) was able to induce cyclin A expression and proliferation in previously non-dividing CLL cells in response to CpG ODN. They

therefore concluded that the magnitude of AKT signalling may determine the 'proliferative capacity' of CLL cells (Longo et al., 2007).

In a subsequent study, Longo and colleagues transfected CLL cells with constitutively active AKT (Myr-AKT) and examined expression of cell cycle related proteins. Active AKT induced substantial increases in the levels of cyclin D3 in all patients, and cyclin E in some patients but not others. They suggested that the difference in cyclin E induction may be due to the 'activation status' of the transfected cells, although definition of the 'activation status' was not clearly explained. Levels of p27, cyclin D2 and cyclin A were unchanged in transfected CLL cells. They therefore concluded that AKT is an important cell cycle regulator in CLL cells, but that AKT alone is insufficient to drive progression of CLL B cells to complete the cell cycle (Longo et al., 2008).

1.4.2.4 Inactivation of AKT

PHLPP phosphatase is an important negative regulator of AKT. It dephosphorylates AKT at serine 473 (Warfel and Newton, 2012, Mendoza and Blenis, 2007). It was shown that protein level of PHLPP1, but not PHLPP2, was significantly reduced in primary CLL cells (Suljagic et al., 2010). It was also shown that enforced expression of PHLPP1 inhibited BCR-induced activation of AKT-GSK3-MCL1 and ERK pathways and reduced survival signalling from other microenvironment stimuli (CD40L, CpG-ODN and CXCL12) in CLL cells. Conversely, down regulation of PHLPP1 in B-cell cell lines increased BCR-stimulation-induced activation of AKT, GSK3 and ERK1/2 kinases (Suljagic et al., 2010). This finding was later independently confirmed by another study where loss of PHLPP1 was observed in over 90% of the CLL samples and its enforced expression inhibited CXCL12-induced AKT and ERK1/2 activation (O'Hayre et al., 2012). The authors found methylation-mediated repression was a potential mechanism for the loss of PHLPP1 in CLL. Together, these studies suggested that reduced expression of PHLPP1 may confer increased responsiveness of CLL cells to BCR, CXCL12 and potentially other signals from the microenvironment, thereby giving the cells survival advantage.

1.4.2.5 Pharmacological inhibition of AKT in CLL

Whilst PI3K inhibitors such as idelalisib and duvelisib have clinical activity in CLL, it is currently unclear whether these effects are mediated via inhibiting AKT. There is some evidence to suggest that apoptosis induction by PI3K inhibitors is mediated via a MAPK pathway rather than by AKT (Rahmani et al., 2003).

There have been a number of studies published exploring the therapeutic potential of pharmacological inhibition of AKT in CLL. AKT inhibitors fall into three categories: ATP competitive AKT kinase domain inhibitors, allosteric PH-domain AKT inhibitors and finally inhibitors that prevent the recruitment of AKT to its signalosome in the lipid raft.

The effects of ATP competitive AKT inhibitors A-443654 (from Abbott Laboratory) and Akti-1/2 (from Merck) have been studied by two independent groups. Both AKT inhibitors induce apoptosis in a dose-dependent manner in primary CLL cells (Zhuang et al., 2010, de Frias et al., 2009). Apoptosis induced by A-443654 was associated with reduced levels of phosphorylated GSK3 α and MCL1. However, both inhibitors (A-443654 and Akti-1/2) were later withdrawn from clinical development due to toxicity caused by many off-target effects.

AiX [10-(4'-(N-diethylamino)butyl)-2-chlorophenoxazine, HCl], another ATP competitive inhibitor of AKT, induced apoptosis in primary CLL cells, mainly from UM-CLL cases which expressed high level of TCL1 (Hofbauer et al., 2010). The apoptotic effect of AiX was also observed in CLL cells co-cultured with stromal cells.

An allosteric AKT inhibitor, MK-2206, was recently developed (Hirai et al., 2010). MK-2206 has been shown to be tolerated in humans but has a long half life and results in maculopapular rash in half of the subjects (Yap et al., 2011). MK-2206 was shown to reduce AKT phosphorylation at 1 μ M concentration, and to induce apoptosis in 50% of primary CLL cells after 72 hours at 8 μ M concentration, but almost no cell death was observed in normal B cells (Ding et al., 2013). *In vitro* MK-2206 synergised with bendamustine and ablated BCR-induced cytokine production. Finally, in an early phase clinical study, MK-2206 showed encouraging activity in eight CLL patients treated (Ding et al., 2013).

Integrin-linked kinase (ILK) activates AKT via phosphorylation on ser 473 (McDonald et al., 2008, Persad et al., 2001). The AKT inhibitor, OSU-T315 was modelled on the scaffold property of the AKT binding site at ILK (Lee et al., 2011). Liu and colleagues showed that OSU-T315 abolished BCR and integrin induced ERK and AKT activation in CLL cells and also inhibited CD40- and TLR9-induced survival signalling (Liu et al., 2015a). OSU-T315 has been shown to prevent the recruitment of AKT to its signalosome in the lipid raft. Preclinical studies showed that OSU-T315 appeared to be effective in primary CLL cells from ibrutinib resistant patients and patients with 17p- and UM-CLL and in the TCL1 mouse model of CLL (Liu et al., 2015a).

Perifosine, an alkyl-phospholipid, inhibits AKT activation. Perifosine decreases the plasma membrane localization of PH-domain containing molecules including AKT (Kondapaka et al., 2003). Initially perifosine was trialled on four patients with CLL who achieved partial responses (Guidetti et al., 2014). However, a phase-II trial in patients with relapsed/refractory CLL was shown to largely induce stabilized disease *in vivo*, through an AKT-independent mechanism (Friedman et al., 2014). Indeed *in vitro* data suggests perifosine appears to have multi-target effects that directly affects the signal transduction pathways involving at least AKT, MAPK, and SAPK/JNK (Li et al., 2006). In addition, *in vitro* study in T-ALL cells suggested perifosine induces apoptosis in a JNK-dependent mechanism (Chiarini et al., 2008).

Given that the PI3K/AKT/mTOR signalling pathway cross-talks with many other signalling pathways, targeting this pathway in combination with others such as the Hedgehog (HH)/GLI pathway may have a synergistic therapeutic effect (Kern et al., 2015). Alternatively, combining with inhibitors such as the BCL-2 inhibitor ABT-199 may improve the efficacy by overcoming drug resistance caused by overexpression of MCL1 and BCL-XL (Choudhary et al., 2015).

1.4.2.6 Other AKT inhibitors

New AKT inhibitors are emerging all the time for use in *in-vitro* assays and in clinical studies (Pal et al., 2010). These include the ATP competitive AKT kinase domain inhibitors, such as GSK690693 (Heerding et al., 2008, Rhodes et al., 2008), GSK2110183 (Afuresertib) and GSK2141795 (Dumble et al., 2014), GDC-0068 (Lin et al., 2013, Blake et al., 2012) and AZD5363 (Addie et al., 2013, Davies et al.,

2012). Other allosteric, MK-2206-like compounds are also in development such as ARQ 092 and ARQ 751. These allosteric inhibitors not only prevent AKT activation by dephosphorylating the membrane-associated active form, but also prevent plasma membrane localisation. In tumour models with an activated AKT pathway, both compounds exhibited strong anti-tumour activity (Yu et al., 2015).

GSK690693 *in vitro* inhibited growth and induced apoptosis in acute lymphoblastic leukaemia cells, non-Hodgkin lymphoma cell lines and Burkitt lymphoma cell lines (Levy et al., 2009). The anti-proliferative effect of GSK690693 was selective for the malignant cells as it did not inhibit the proliferation of normal cells. GSK690693 also delayed tumour progression in a mouse model with constitutively active AKT (Altomare et al., 2010). Whilst GSK690693 demonstrated broad activity *in vitro*, results against solid tumour *in vivo* demonstrated that as a single agent GSK690693 had only modest antitumor activity at the dose and schedule used (Carol et al., 2010). Since GSK690693 was not orally bioavailable it has been superseded by two other GSK compounds, GSK2110183 (Afuresertib) and GSK2141795, which are orally bioavailable, potent inhibitors of the AKT kinases that have progressed to human clinical studies (Dumble et al., 2014). Afuresertib (GSK2110183) has been used in early phase clinical trials in patients with multiple myeloma (Spencer et al., 2014) and in solid tumours (Tolcher et al., 2015), with some promising results.

GDC-0068 at clinically achievable doses inhibited tumour growth in preclinical models, however, in clinical study a compensatory feedback activation of ERK and HER3 was observed (Yan et al., 2013).

AZD5363 has been shown to be useful for treatment of cancers with *PI3KCA* mutations or PTEN loss, such as breast cancer (Davies et al., 2012) and gastric cancers with *PI3KCA* mutations (Li et al., 2013). AZD5363 was well tolerated in humans and yielded partial responses in patients with advanced solid tumours (Banerji, 2013). AZD5363 may be useful in tumours that contain AKT1 (E17K) mutations since two patients with breast and ovarian tumours containing AKT1 mutations showed partial responses to AZD5363 (Davies et al., 2015). However, pre-clinical models suggest that AZD5363 will most likely be used in combination with other inhibitors in cancers, such as in combination with the FGFR inhibitor AZD4547 in bladder cancer (Davies et al., 2015) or in combination with

salinomycin, Fulvestrant (ICI182780) or AZD8931 in breast cancers (Choi et al., 2015, Ribas et al., 2015, Crafter et al., 2015). In non-small cell lung cancer cell lines AZD5363 synergised with gefitinib (Puglisi et al., 2014) and in acute myeloid leukaemia cell lines AZD5363 synergised with PIM inhibitors (Meja et al., 2014).

AZD5363 in combination with enzalutamide (ENZ), a hormone therapy treatment for castrate-resistant prostate cancer (CRPC), showed increased apoptosis in a pre-clinical model (Toren et al., 2015). AZD5363 has been observed to induce autophagy in some prostate cancer cells through downregulation of the mTOR pathway and therefore it has been hypothesised that addition of autophagy inhibitors may improve efficacy (Lamoureux et al., 2013).

All AKT inhibitors seem to induce hyperglycaemia, although this side effect is clinically manageable either by fasting before drug administration or by following a low carbohydrate diet after administration (Crouthamel et al., 2009).

1.5 Hypothesis & Aim

Given the important role of PI3K and AKT in mediating survival signals from the CLL microenvironment, I hypothesise that PI3K-dependent activation of AKT mediates both the cytoprotective and proliferative effects of CD40 stimulation in the presence of IL-4/IL-21 in primary CLL cells. To test this hypothesis, I set out to address the following research questions:

1. To what extent does AKT contribute to the survival of CLL cells cultured under conditions that mimic the lymph node microenvironment?
2. To what extent does AKT contribute to the proliferation of CLL cells induced by CD154 + IL-4/IL-21?
3. To what extent does PI3K regulate activation of AKT in CD40-stimulated CLL cells?

Thus, my overall aim is to gain better understanding on the role and regulation of AKT in CLL-cell survival and proliferation in response to stimuli relevant to the lymph node microenvironment.

Chapter 2 : Methodology

2.1 Methods

2.1.1 Cell preparation

The major disadvantage of using one of the very few *bona fide* cell lines to study CLL is that they do not represent the heterogeneity observed in CLL. Secondly, the fact that all lymphoblastoid cell lines contain the Epstein Barr Virus (EBV), but that EBV is not normally involved in the pathogenesis of CLL, raises questions about their validity. Whilst immortalized CLL-derived cell lines can be a valuable source to study antibody specificities and genetic (DNA) aberrations, inferences based on factors responsible for survival and proliferation of CLL cells cannot be made due to EBV imprinting in these cell lines (Lanemo Myhrinder et al., 2013). Finally, cell lines do not display dependence on exogenous signals from the microenvironment to support cell survival and proliferation, whereas CLL cells normally do (Burger, 2011, Caligaris-Cappio et al., 2014). In addition, Longo and colleagues compared cellular responses to activation of BCR by antigen stimulation between primary CLL cells and several B cell lines and showed that some B cell lines behave differently to primary CLL samples, partly due to the differential expression of apoptosis-regulatory proteins (Longo et al., 2008).

With these considerations in mind, throughout my PhD study I only used primary CLL cells in the experiments. I therefore cultured primary CLL cells under standard conditions or co-cultured them with mouse fibroblasts that were stably transfected to express human CD154 to mimic the *in-vivo* interaction of T cells with CLL cells involving the CD40-CD154 signalling pathway in the lymph node microenvironment.

2.1.1.1 CLL cell preparation

All CLL samples were obtained with informed consent and with the approval of the Liverpool Research Ethics Committee (The REC reference of the Biobank is 06/Q1505/81. The REC reference for the project is 06/Q1505/82). The diagnosis of CLL was based on standard morphological and immunophenotypic criteria (Swerdlow S, 2008). Peripheral blood mononuclear cells (PMBCs) were prepared from CLL patients and healthy volunteers by centrifugation of blood over

Lymphoprep (Cat. no. 1114544, Axis-Shield PoC AS, Oslo, Norway), as indicated in Figure 2.1. In most experiments, PBMCs were stored at -150°C prior to use. Clinical and laboratory characteristics of the CLL samples that I studied are summarised in Table 2.1.

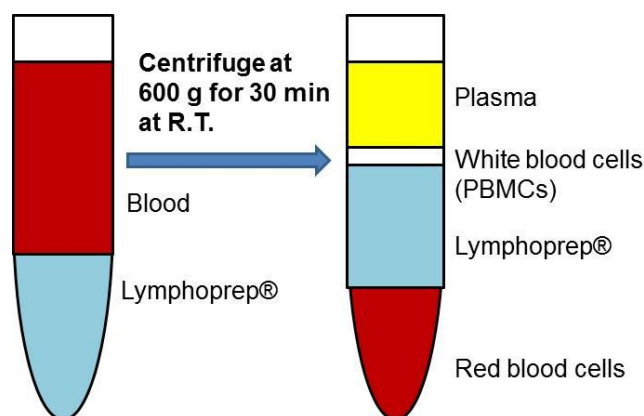


Figure 2.1: Peripheral blood mononuclear cells (PBMC) preparation

Peripheral blood mononuclear cells (PBMC) were isolated from patients and healthy volunteers by centrifugation of blood over Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) at 600g for 30 min at room temperature.

2.1.1.2 Purification of CLL B cells

In cases where applicable, CLL B cells were further purified from CLL PBMCs using a commercially available negative selection kit, the B-CLL Cell Isolation Kit (Cat. no. 130-103-466, Miltenyi Biotec Ltd, Surrey, UK). Non B-CLL cells are labelled with a cocktail of biotin-conjugated antibodies against CD2, CD3, CD4, CD14, CD15, CD16, CD56, CD61, CD235a, Fc ϵ RI and CD34, which are subsequently counter-labelled with magnetic anti-biotin MicroBeads. Isolation of unlabelled B cells is achieved by depletion of magnetically labelled cells. The B-CLL cell purity was assessed by flow cytometry for CD5 & CD19 double positivity, using CD19-PerCPCy5.5 and CD5-PE antibodies, as shown in Figure 2.2. The percentage of CD5 & CD19 double positivity of CLL cells pre- and post-purification is given in Table 2.2. For more specific details regarding the isolation protocol, see Appendix section 1.1 Purification of CLL B cells.

Table 2.1: Primary CLL cells

¶ IGHV status refers to somatic mutation in IGHV gene of CLL cells as compared with the gene sequence of the nearest germ-line using 2% as a cut-off. M=mutated; UM=un-mutated.

Sample number	Patient number	Gender	Age at diagnosis	Rai Stage	Binet Stage	Patient Status	WBC (10 ⁹ /l)	Lymphocyte count (10 ⁹ /l)	p53	IGHV status ¶	% VH	VH Gene Usage	IGVH Class	Chromosomal abnormalities
1958	275	F	65	0	A	A	215		WT	M	4.47	4-43/DP-63	M	13q-
2064	280	M	46			D	120		B	UM	0.33	3-15/DP-38	G	Tri 12 (80%)
2096	325	M	65	0	A	A	60	52.6	B	M	6.25	3-7*01	M	17p- (21%)
2103	519	M	56			D	125	119	A	UM	0	1-69/DP-10	M	17p- (95%)
2263	388	M	66			D	366.5		B	UM	0.68	1-69/Yac-7	M	11q- (97%)
2441	404	M	44		A	A	153	137	B	UM	1.75	4-59*01	M	Normal
2474	143	M				A	108.6	104.1	B	UM	0	DP8+	M	11q- (96%)
2521	111	F	80		B	D	73.6	61.6	WT	M	10.47	3-30/DP-49	M	Tri 12
2674	104	F	47	III	B	D	42.2	36.1	B	UM	0	3-64*01	M	17p- (90%)
2729	187	M	67	0	A	A	37.2	30.6		M	8.5	3-72*01	M	Normal
2814	283	M	72			A	196.1			UM	0	4-34*01	M	Normal
3033	187	M	67	0	A	A	41.3	34.9		M	8.5	3-72*01	M	Normal
3058	493	M	70		B	A	51.3	48.4		UM	0	1-2*04	M	Normal
3074	172	M	78		A	A	459.7	225.7	WT	UM	0.34	3-49*03	M	13q- (30.37%)
3091	150	M				A	41.7	38.2		UM	0			
3106	221	M	66	IV	C	A	115	103		UM	0	1-69*01	M	
3129	150	M				A	31.8	28.9		UM	0			
3308	221	M	66	IV	C	A				UM	0	1-69*01	M	
3325	106	F		IV	C	A	171.4			M	5.21	3-23*01	M	17p- (77%)
3347	99	F	53	III		D	115	104						
3353	203	F	75	0	A	A	72	121.7		M	11.58	4-34*02	G	
3354	332	F	61	I	B	A	70.5	59.9		UM	0	4-59*	M	
3355	307	M	73	I	B	A	130.2	121.7		M	3.4	7-04.1/VI-4.1	M	13q- (74%)
3357	488		79	II	B	A	20	14		UM	0	5-51*01	M	
3361	383	M	68	II	B	A	172.3	165.8		UM	0.35	3-74*01	M	17p-
3363	24	M				A			WT	UM	0.35	3-48*01	M	Normal
3365	29	F		0	C	A	253	249						

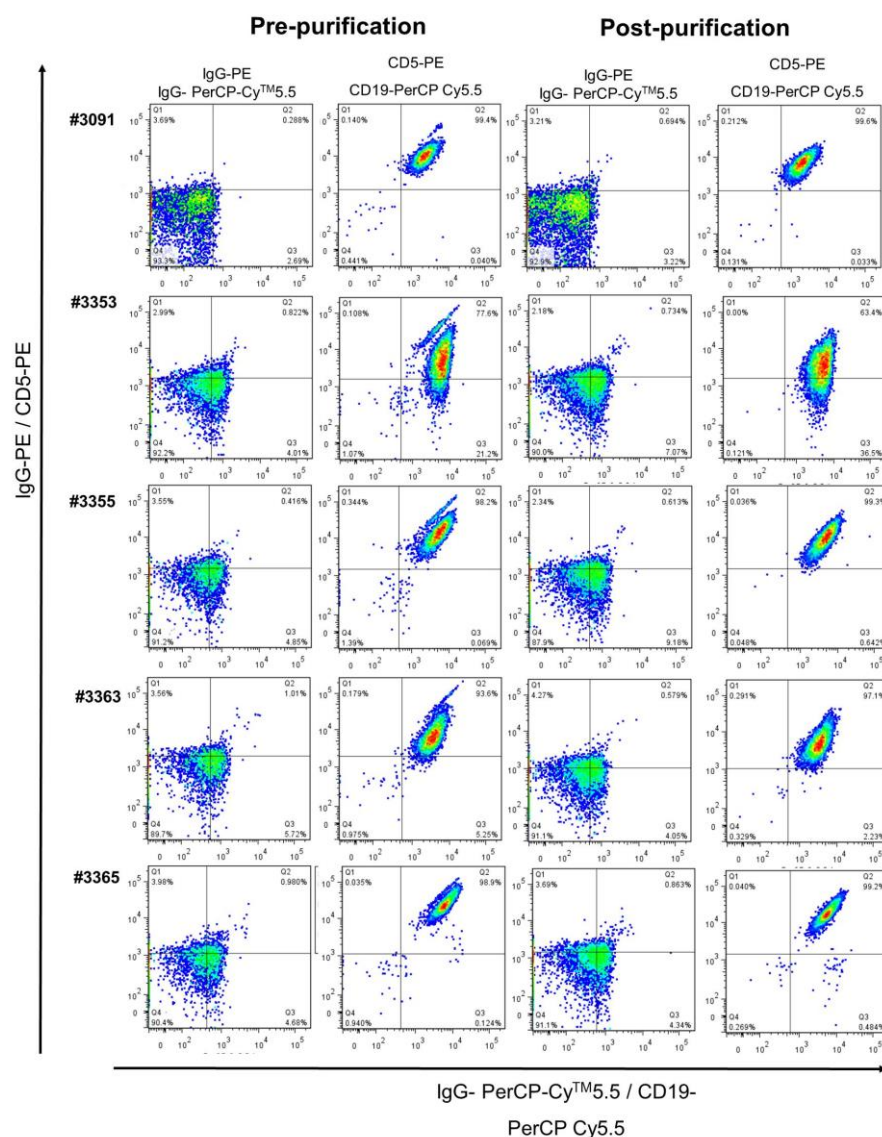


Figure 2.2: Purified CLL cells.

CLL PBMCs were purified using B-CLL Cell Isolation Kit, human (130-103-466, Miltenyi Biotec Ltd). The B-CLL cell purity was assessed by CD19, CD5 positivity, using CD19-PerCPCy5.5 and CD5-PE antibodies and control- PerCP-CyTM5.5, PE antibodies on the BD FACSCaliburTM. Quadrant plots showing CD5+ CD19+ cells are displayed pre and post-purification.

Table 2.2: Purified CLL cells.

CLL PBMCs were purified using B-CLL Cell Isolation Kit, human (130-103-466, Miltenyi Biotec Ltd). The B-CLL cell purity was assessed pre and post-purification by CD19, CD5 positivity, using CD19-PerCPCy5.5 and CD5-PE antibodies. This table displays % CD5+ CD19+ cells.

	% CD5+ CD19+	
	Pre-purification	Post-purification
3091	99.4	99.6
3353	77.6	63.4
3355	98.2	99.3
3363	93.6	97.1
3365	98.9	99.2

2.1.1.3 Normal B cells isolated from healthy donors

Normal B cells were further isolated from the PBMCs obtained from healthy donors, as described earlier, using a commercially available negative selection kit, the B Cell Isolation Kit II (Cat. no. 130–091–151, Miltenyi Biotec ltd). Briefly, non-B cells (T cells, NK cells, monocytes, dendritic cells, granulocytes, platelets, and erythroid cells) are labelled with a cocktail of biotinylated CD2, CD14, CD16, CD36, CD43, and CD235a (glycophorin A) antibodies, which are subsequently magnetically labelled with anti-biotin MicroBeads for depletion. Purified normal B cells are obtained by depletion of magnetically labelled non-B cells, as depicted in Appendix Figure 1. B cell purity was assessed by CD19 positivity, using CD19-PE antibody and isotype control-PE antibodies on FL-2 of the flow cytometer, as described in Figure 2.3. Details of the normal B cells isolated from the healthy donors are given in Table 2.3. For more specific details regarding the isolation protocol, see Appendix section 1.2 Normal B cells isolated from normal healthy donors.

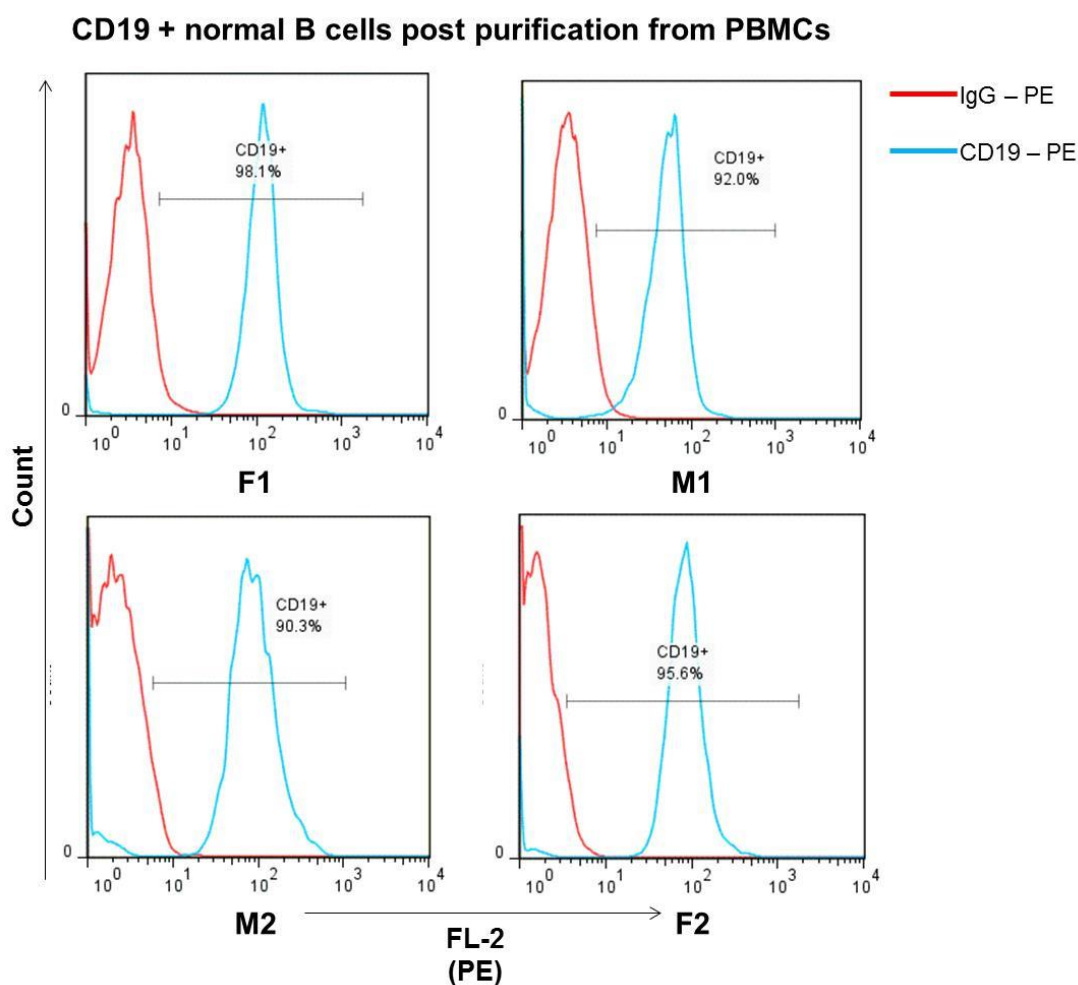


Figure 2.3: Measuring CD19+ cells as a marker of normal B cells by flow cytometry

Normal B cells from PBMCs of four healthy donors were isolated using B Cell Isolation Kit II (Miltenyi Biotec Ltd, Surrey, UK). The B cell purity was assessed by CD19 positivity, using CD19-PE antibody and control-PE antibodies and analysed by flow cytometry. Overlay of CD19-PE and control-PE plots shown, showing % of cells that are CD19 positive.

Table 2.3: Normal B cells isolated from healthy donors

Donor number	Code	Gender	Age	CD19+ cells (%)
#1	F1	F	27	98.1
#2	M1	M	50	92.0
#3	F2	F	31	90.3
#4	M2	M	40	95.6

2.1.1.4 Normal B cells isolated from buffy coats

In order to collect sufficient numbers of normal B cells for proliferation assay, buffy coats were purchased from NHS Blood and Transplant (Speke, Liverpool, UK). The PBMCs from the buffy coats were prepared by centrifugation of blood over Lymphoprep as described in section 2.1.1.1 CLL cell preparation.

Normal B cells from the PBMCs were isolated using the B Cell Isolation Kit II, as previously described. The B cell purity was assessed by CD19 positivity on flow cytometry using CD19-PE antibody and control-PE antibodies, as shown in Figure 2.4. Details of the normal B cells isolated from buffy coats regarding the percentage of CD19 positivity and their use are summarised in Table 2.4.

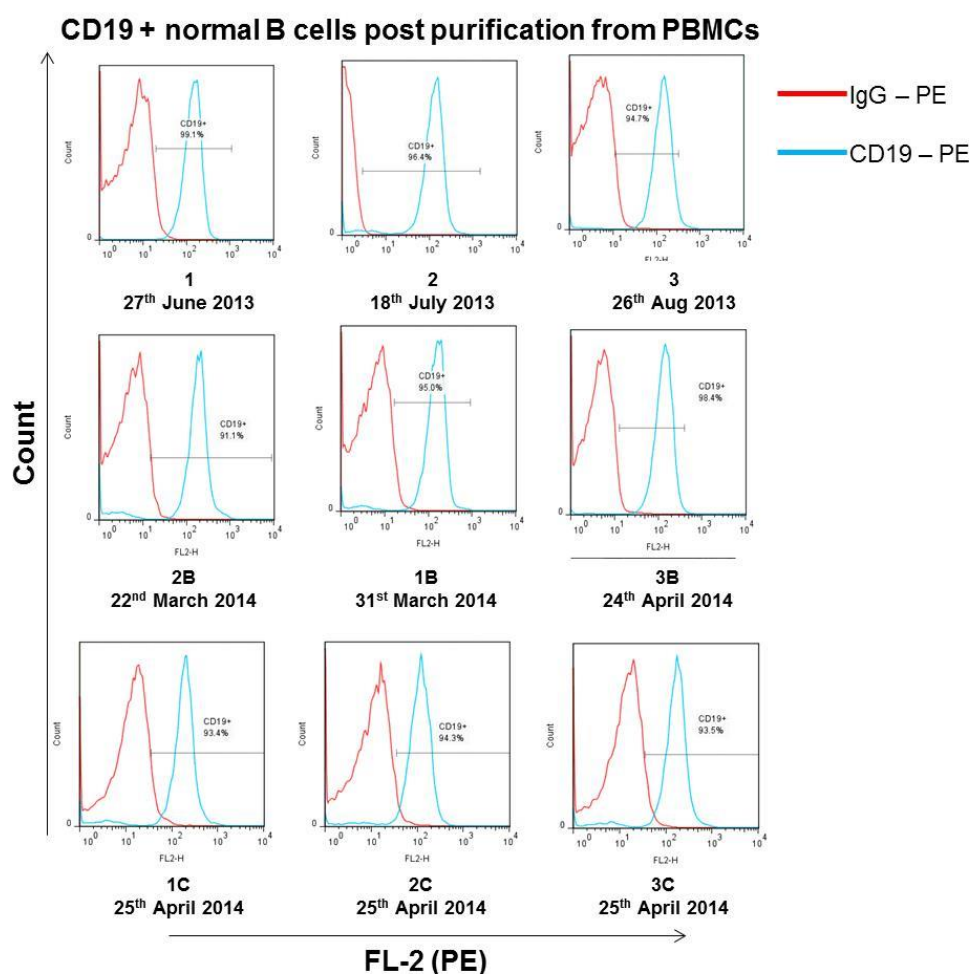


Figure 2.4: CD19 + normal B cells post purification from PBMCs of buffy coats

Buffy Coat B cells were purified using B Cell Isolation Kit II. The B cell purity was assessed by CD19 positivity, using CD19-PE antibody and control-PE antibodies on FL-2 on the BD FACSCalibur™. The % of CD19+ cells is displayed.

Table 2.4: Normal B cells isolated from buffy coats

Buffy coats	CD19+ QC (%)	What used for
1	99.1%	CD154 + IL-4 proliferation assay (1)
2	96.4%	CD154 + IL-4 proliferation assay (2)
3	94.7%	CD154 + IL-4 proliferation assay (3)
2B	91.1%	CD154 + IL-21 proliferation assay (1)
1B	95.0%	CD154 + IL-21 proliferation assay (2)
3B	98.4%	CD154 + IL-21 proliferation assay (3)
#3397 – 1C	93.4%	Western blotting
#3398 – 2C	94.3%	Western blotting
#3399 – 3C	93.5%	Western blotting

2.1.2 Culturing primary CLL cells under standard conditions

CLL cells were essentially maintained in RPMI-1640 medium (#R0883, Sigma, Dorset, UK) supplemented with 10% heat-inactivated fetal bovine serum (#F9665, Sigma), 2 mM L-glutamine (#G4513, Sigma) and 1% penicillin/streptomycin (#P0781, Sigma).

Cryopreserved CLL cells were first thawed at 37°C in a water-bath and returned on ice and mixed, drop-by-drop, with 10 ml of the above RPMI medium in a sterile 20 ml universal tube. Thawed cells were spun at 550g for 5 min at 4°C, washed in 10 ml of the RPMI medium, and finally re-suspended in 2-5 ml of the RPMI medium and kept in a 5% CO₂ incubator at 37°C for 1 hour to recover.

The viability was measured by trypan blue exclusion using a Cellometer (Cellometer Auto T4 Cell Viability Counter, Nexcelom Bioscience, MA, USA). Cells were usually cultured at 4-5 x 10⁶ cells/ ml in the RPMI medium in a 5% CO₂ incubator at 37°C.

2.1.3 Co-culture of CLL cells

2.1.3.1 Source and maintenance of parental and CD154-expressing mouse fibroblasts

Stably transfected mouse fibroblasts, both parental cells which were transfected with an empty vector and hence used as control, and those that were transfected with an expression vector containing human CD154 cDNA so the transfected fibroblasts constantly express human CD154 on the cell surface, were provided by Professor Gerry Cohen at the University of Liverpool and maintained as described by Vogler (Vogler et al., 2009a).

The fibroblasts were routinely maintained in DMEM medium (high glucose) (#D6546, Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin in a 5% CO₂ incubator at 37°C.

2.1.3.2 Co-culture of CLL cells with CD154-expressing fibroblasts

To mimic the *in-vivo* interaction of T cells with CLL cells via the CD154-CD40 signalling pathway in the lymph node microenvironment, CLL cells were cultured on

an adherent monolayer of γ -irradiated fibroblasts expressing CD154 or control fibroblasts at a ratio of 10 to 1 and maintained at 37°C for an appropriate period of time. To determine the effect of CD40 ligation on killing induced by bendamustine or AZD5363, CLL cells were co-cultured with the CD154-expressing or control fibroblasts for 24h or 48h in the presence or absence of the above drugs before harvesting (for detailed description of co-culture, see Appendix 1.3 Co-culture of CLL cells with CD154-expressing fibroblasts).

On harvest, CLL cells were collected by gentle pipetting (to avoid disrupting the monolayer of fibroblasts) for downstream analysis (e.g. Western blotting or FACS analysis).

The effect of on CD40L expression in the fibroblasts was not evaluated upon co-culture with CLL cells or with the various drugs.

2.1.4 Proliferation assay

There are many different ways of measuring proliferation, each with its own advantages and disadvantages (see summary in Table 2.5). In my PhD study, cell division was measured using cell labelling with a fluorescent dye, CFSE, and DNA synthesis was measured by the BrdU incorporation assay.

2.1.4.1 Measuring cell division using CFSE

CFSE was first used to measure cell division of T lymphocytes (Quah et al., 2007). The principle behind its use is that the fluorescence intensity of the dye reduces by half in daughter cells after each cell division (Parish, 1999).

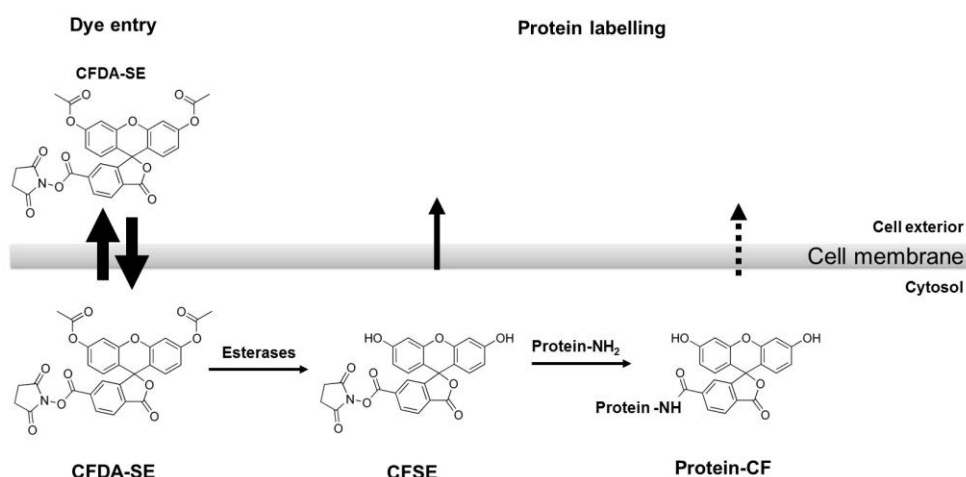
Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) is the CFSE precursor that is used to label cells. CFDA-SE is non-fluorescent, but highly membrane permeable. Upon entering the cells, acetate groups are removed by intracellular esterases, causing the resulting CFSE molecule to become fluorescent. CFSE is less membrane permeable inside the cells and the succinimidyl ester group of CFSE covalently binds to primary amine groups, thus remaining bound to proteins inside cells for long time periods (Figure 2.5A). As a cell divides, the intensity of CFSE fluorescence in the resultant daughter cells will be half that of the parent cell, allowing easy monitoring by flow cytometry on the number of cell divisions that

have occurred since labelling. CFSE has similar excitation and emission properties as fluorescein isothiocyanate (FITC), thus CFSE can be assayed in flow cytometry by the same channels that detect the fluorescence intensity of FITC. Sophisticated mathematical models have also been developed to analyse CFSE data, such as FlowJo software (Figure 2.5B-C).

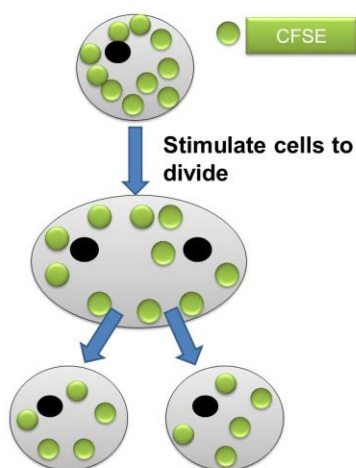
Table 2.5: Methods of measuring proliferation

Measuring	Name of assay	Description
DNA synthesis	Tritiated thymidine (^3H).	radioactively labelled thymidine (^3H). Disadvantage: Radioactive.
	BrdU	nucleotide analogue 5-bromodeoxyuridine (BrdU)
	EdU	EdU, a new thymidine analogue
Mitochondrial dehydrogenases (enzymatic assays)	MTT-test	The enzymatic assays evaluating the turnover of mitochondrial dehydrogenases
Lysosomal hexosaminidase (enzymatic assays)	NAG-test	The enzymatic assays evaluating the turnover of lysosomal hexosaminidase
Metabolism	Alamar Blue ®	alamarBlue® is intermediate only between final reduction of O_2 and cytochrome oxidase (Cyt.a3).
Cyclins/ proliferating cell nuclear antigen (PCNA)/ immunostaining	Immunostaining	For marking S phase cells in paraffin sections from animal and human tissues
	Ki67	The fact that the Ki-67 protein is present during all active phases of the cell cycle (G(1), S, G(2), and mitosis), but is absent from resting cells (G(0)), makes it an excellent marker for determining the so-called growth fraction of a given cell population.
Mitosis / Cell division	CFSE	Dilution dye assay, allows for discrimination between subsequent cell divisions

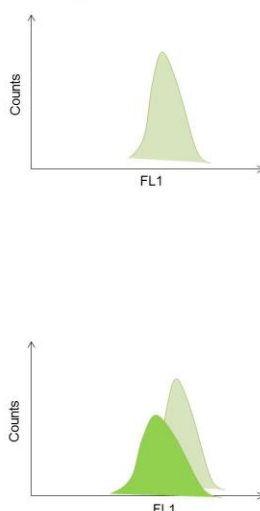
A. Schematic representation of the mechanism of fluorescently labelling cells with carboxyfluorescein diacetate succinimidyl ester.



B. Schematic of how CFSE labelling can be used to measure mitosis



C. Decreases in the FL1 peak shift



D. Real CFSE FL1 data

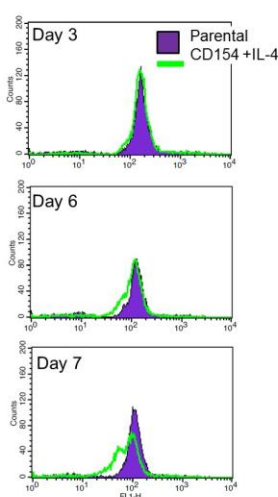


Figure 2.5: CFSE labelling to measure cell division

A. Schematic representation of the mechanism of fluorescently labelling cells with carboxyfluorescein diacetate succinimidyl ester.

Initially, the non-fluorescent, highly membrane permeant, 6-carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) is readily taken up by cells, although its high lipophilicity also allows it to freely exit from cells. Intracellular esterases, remove the two acetate groups from CFDA-SE to yield fluorescent 6-carboxyfluorescein succinimidyl ester (CFSE), which is much less membrane permeant and, therefore, exits from cells at a much slower rate. The succinimidyl moiety of CFSE reacts with amino groups of proteins resulting in 'Protein-CF' molecules. Most of the molecules cannot escape from the cell so stable fluorescent labelling of cells is achieved. The arrow size indicates the diffusion rate of the different molecules across the cell membrane. Adapted from (Parish, 1999).

B. Schematic diagram showing how CFSE labelling can be used to measure cell division.

CFSE enters the cytoplasm of cells, in this figure ten molecules. Upon stimulation to divide, the cells initially grow and the DNA is replicated. When mitosis occurs, the original molecules of CFSE are halved between the two daughter cells, in this figure five molecules in each daughter cells.

C. Decreases in the FL1 peak shift

CFSE enters the cytoplasm of cells and is retained within cells for long periods. CFSE can be used in vitro to measure lymphocyte proliferation, due to the progressive halving of CFSE. When mitosis occurs, the fluorescence within daughter cells following each cell division halves. The CFSE fluorescence can be measured by FACS using the FL1 channel. The FL1 peak shifts to the left upon cell division.

D. Real CFSE FL1 data from CLL cells stimulated with CD154 + IL-4, day 3, day 6 and day 7.

2.1.4.2 Induction of proliferation of CLL cells by CD40 + IL-4 / IL-21

To induce CLL-cell proliferation by CD40 + IL-4 / IL-21, CLL cells were first labelled with CFSE dye (for detailed description, see Appendix 1.4. CFSE labelling of CLL cells). After final washing, labelled CLL cells were re-suspended in fresh RPMI medium supplemented with either 10 ng/ml recombinant human IL-4 (#204-IL-010, R&D, Abingdon, USA) or 15 ng/ml recombinant Human IL-21 (Cat. no. PHC0215, Life Technologies) prior to being plated onto γ -irradiated control and CD154-expressing fibroblasts in a multi-well plate. At day 3/4 and day 7 the culture medium was partially replaced with fresh complete RPMI medium containing 10 ng/ml IL-4 or 15 ng/ml IL-21. At the end of co-culture, CLL cells were harvested and analysis was carried out using a flow cytometer.

2.1.5 BrdU incorporation assay to measure DNA synthesis

DNA synthesis was measured by BrdU incorporation using a commercial kit (Cell Proliferation ELISA, BrdU (colorimetric), Cat. no.11647229001, Roche via Sigma), as per the manufacturers protocol (Figure 2.6). Briefly, CLL cells were co-cultured with CD154-expressing fibroblasts in the presence of IL-21 and with drugs at the indicated concentrations in a 96 well plate for 24 hours. BrdU labelling solution was then added to the cell culture. 48 hours later, cells were fixed and denatured, incubated with anti-BrdU-peroxidase solution and washed three times with PBS. The substrate reaction was allowed to proceed for between 5-30 minutes, then 1M H₂SO₄ stop solution was used to neutralise the reaction. The plates were read at 450nm.

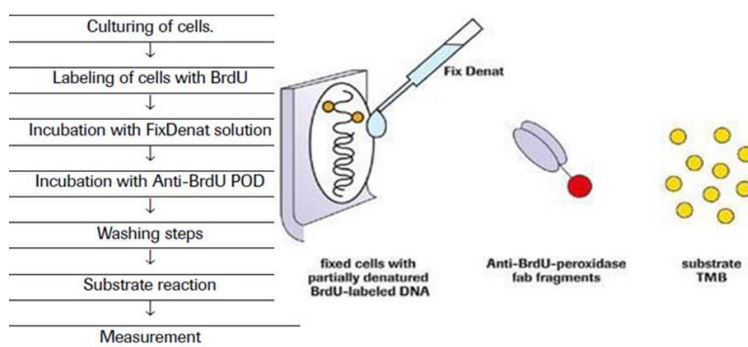


Figure 2.6: DNA synthesis was measured using BrdU assay

Briefly, CLL cells were co-cultured with CD154-expressing fibroblasts in the presence of IL-21 and drugs at the indicated concentrations in a 96 well plate for 24 hours. BrdU labelling solution was added on day 1 after initiation of co-culture. On day 3, cells were fixed and denatured, incubated with Anti-BrdU-peroxidase solution, washed three times with PBS and the substrate reaction was allowed to proceed for between 5-30 minutes, then 1M H₂SO₄ Stop Solution was used to neutralise the reaction.

2.1.6 shRNA lentiviral production

Currently, there are two commonly used methods to silence gene expression by RNA interference (RNAi) in mammalian cells, the use of siRNA and shRNA. The strengths and limitations of each approach have been well documented in the review by Moffat and Sabatini (Moffat and Sabatini, 2006) and are summarised in Figure 2.7. Previous work from this laboratory and others has shown that transfecting primary CLL cells with siRNA is difficult mainly due to the non-dividing nature of primary CLL cells *in vitro*. Therefore, I tried the lentiviral delivery of shRNA approach, making use of the shRNA Bacterial Glycerol Stock library purchased from Sigma-Aldrich Ltd. by the University, which was housed in the Department. The work flow of the lentiviral delivery of shRNA is given in Figure 2.8. The production of lentiviral particles containing shRNAs of interest and transfection work were performed by Dr Mark Glenn to comply with biosafety regulations of the University (for detailed description of lentiviral infection of CLL cells, see Appendix 1.5 shRNA lentiviral production).

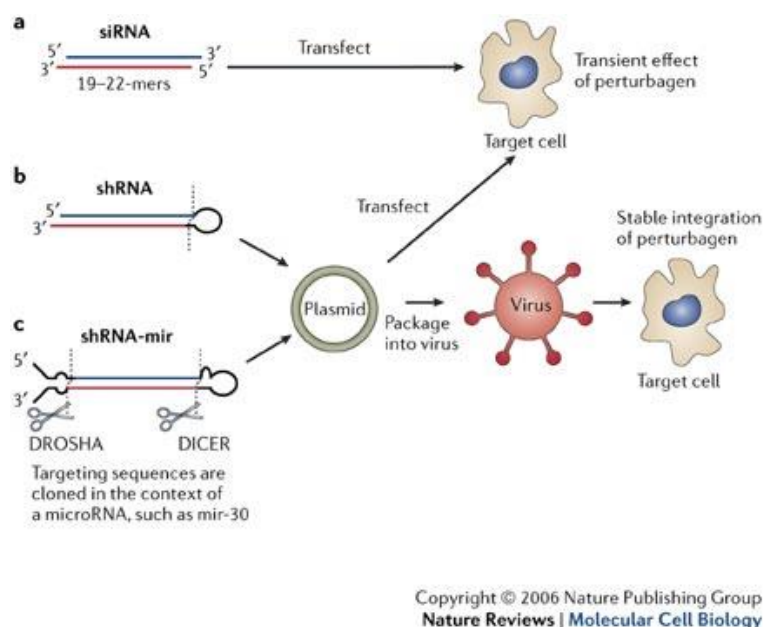


Figure 2.7: Knock down approaches: shRNA versus siRNA

a) Small interfering RNAs (siRNAs) are short double-stranded RNAs that are typically 19–22 bp in length. siRNAs can be made by chemical synthesis and purchased from biotechnology companies. They can be introduced into target cells by transfection to cause transient silencing of a target gene.

b/c) Short hairpin RNAs (shRNAs) or shRNAs in a microRNA context (shRNA-mirs) are constructed in a plasmid backbone and can be transfected or packaged into a virus and transduced into target cells. Viral transduction results in the stable integration and expression of an shRNA or shRNA-mir in the target cell.

All three of these types of small RNAs can be used to silence gene function in mammalian cells. Figure taken from (Moffat and Sabatini, 2006).

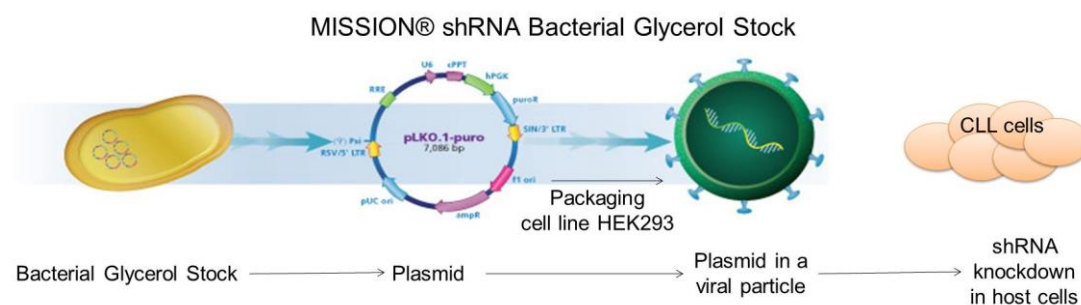


Figure 2.8: The work flow of the lentiviral delivery of shRNA in CLL cells

The Bacterial Glycerol Stock contains plasmids containing shRNAs encoded in a plasmid. The bacteria containing the choice plasmids are grown up, and the plasmids extracted. The transfer plasmid containing the shRNA of interest (pLKO.1-puro) or pLKO.1-puro-CMV-TurboGFP and plasmid psPAX2, and plasmid pMD2G are then transfected into a packaging cell line HEK293, to generate plasmid in viral particles. The amount of viral particle can then be quantitated using qRT-PCR, before infecting into host cells, in our case CLL cells.

2.1.6.1 shRNA clones used

The following shRNA clones were examined:

Table 2.6: shRNA clones

Target	Clone ID	TRC Version	TRC Number	TRC Number	Sequence of shRNA	Alternate Species
AKT1						
AKT1 (SHCLNG-NM_005163)	NM_005163.x-1044s1c1	TRC 1	TRCN0000010162	10162	CCGGGGACAAG GACGGGCACATT AACTCGAGTTAA TGTGCCCCGTCCT TGTCCTTTTT	NM_005163.2, Homo sapiens v- akt murine thymoma viral oncogene homolog 1
AKT1 (SHCLNG-NM_005163)	NM_005163.x-642s1c1	TRC 1	TRCN0000010163	10163	CCGGCGAGTTTG AGTACCTGAAGC TCTCGAGAGCTT CAGGTACTCAAA CTCGTTTTT	(AKT1), transcript variant 1, mRNA NM_001014431.1
AKT1 (SHCLNG-NM_005163)	NM_005163.x-981s1c1	TRC 1	TRCN0000010174	10174	CCGGGGACTACC TGCACTCGGAGA ACTCGAGTTCTC CGAGTGCAGGTA GTCTTTTTT	Homo sapiens v- akt murine thymoma viral oncogene homolog 1
AKT1 (SHCLNG-NM_005163)	NM_005163.1-735s1c1	TRC 1	TRCN0000039794	39794	CCGGGATCCTCA AGAAGGAAGTC ATCTCGAGATGA CTTCCTTCTTGA GGATCTTTTTG	(AKT1), transcript variant 3, mRNA NM_001014432.1
AKT1 (SHCLNG-NM_005163)	NM_005163.1-628s1c1	TRC 1	TRCN0000039797	39797	CCGGCGCGTGAC CATGAACGAGTT TCTCGAGAACT CGTTCATGGTCA CGCGTTTTTG	Homo sapiens v- akt murine thymoma viral oncogene homolog 1 (AKT1), transcript variant 2, mRNA
AKT2						
AKT2 (SHCLNG-NM_001626)	NM_001626.x-354s1c1	TRC 1	TRCN0000000563	563	CCGGCCCTTAAA CAACTTCTCCGT ACTCGAGTACGG AGAAGTTGTTTA AGGGTTTTT	NM_001626.2 [Homo sapiens v-akt murine thymoma viral oncogene homolog 2 (AKT2), mRNA]
AKT2 (SHCLNG-NM_001626)	NM_001626.2-1509s1c1	TRC 1	TRCN0000039968	39968	CCGGACAAGGTA CTTCGATGATGA ACTCGAGTTCAT CATCGAAGTACC TTGTTTTTTG	
AKT2 (SHCLNG-NM_001626)	NM_001626.x-656s1c1	TRC 1	TRCN0000000564	564	CCGGCTTCGACT ATCTCAAACCTCC TCTCGAGAGGAG TTTGAGATAGTC GAAGTTTTT	
AKT2 (SHCLNG-NM_001626)	NM_001626.x-632s1c1	TRC 1	TRCN0000000565	565	CCGGACGGGCTA AAGTGACCATGA ACTCGAGTTCAT GGTCACTTTAGC CCGTTTTTT	
AKT2 (SHCLNG-NM_001626)	NM_001626.x-355s1c1	TRC 1	TRCN0000000566	566	CCGGCCCTTAAAC AACTTCTCCGTA GCTCGAGCTACG GAGAAGTTGTTT AAGGTTTTT	

2.1.6.2 Infection of CLL cells with lentiviral particles

Figure 2.9 illustrates a schematic diagram of non-replicating lentiviral delivery of stable shRNA expression in CLL cells. The day prior to infection, CD154-expressing fibroblasts were irradiated and plated out into 6 well plates as previously described. CLL cell infection was carried out using a spinoculation procedure. Briefly, CLL cells were plated out at 7×10^6 cells/ml in 24 well plates, to which the concentrated

lentivirus particles were added. The plates were then centrifuged at 1200g for 2 hours at 25°C. Finally, the CLL cells were re-suspended in the infection medium and transferred to the 6-well plates containing CD154-expressing fibroblasts. Following 48-72 hours incubation, CLL cells were harvested and, where applicable, GFP expression of infected cells was analysed for infection efficiency on flow cytometry. Otherwise cells were lysed, protein concentration determined and Western blotting analysis was performed to examine the expression of AKT1 and AKT2. Figure 2.10 summarises the above steps of lentiviral infection procedure in CLL cells.

It is well appreciated that high titres of virus are needed when infecting primary CLL cells. Cantwell and colleagues showed using adenovirus that the efficiency of gene transduction into CLL B cells was approximately 100 to 1,000-fold lower than into HeLa cells at any given multiplicity of infection (MOI). They found that at a MOI of 500, 10% to 70% of the CLL B cells from different patients were made to express the transgene, as assessed by multiparameter flow cytometric analysis (Cantwell et al., 1996). Others using similar viral systems with CLL cells find similar results (Andrew Steele, personal correspondence). In my experiments I used an MOI of 150×10^3 , 1.5×10^5 LV particles/CLL cells (see [appendix section 1.5.2](#), [1.5.3](#) and [1.5.4](#) and Figure 4.26F for further information). The titre of virus was measured using qRT-PCR of the viral *gag* gene ([appendix section 1.5.4](#)). This gene is encoded on the packaging psPAX2 plasmid. This gene codes for the core structural proteins of a retrovirus. It is possible for the qRT-PCR to therefore give false positives, since it is possible for the virus to be constructed without including shRNA expression cassette. In fact others find that as many as two in three virions are “empty viruses” when using this measurement (Andrew Steele, personal correspondence). One way of defining viral titre and working out if the virus is active, would’ve been to include an expression protein that could be assessed via flow cytometry or western blot such as GFP protein, which was used however it was not readily expressed (Figure 4.26D), suggesting that the virus’ may be empty virus. Alternatively qRT-PCR to the cassette of interest could be used. There may also be the problem that the CLL cells may not infect efficiently and experiments should first be performed in an easy to infect cell line.

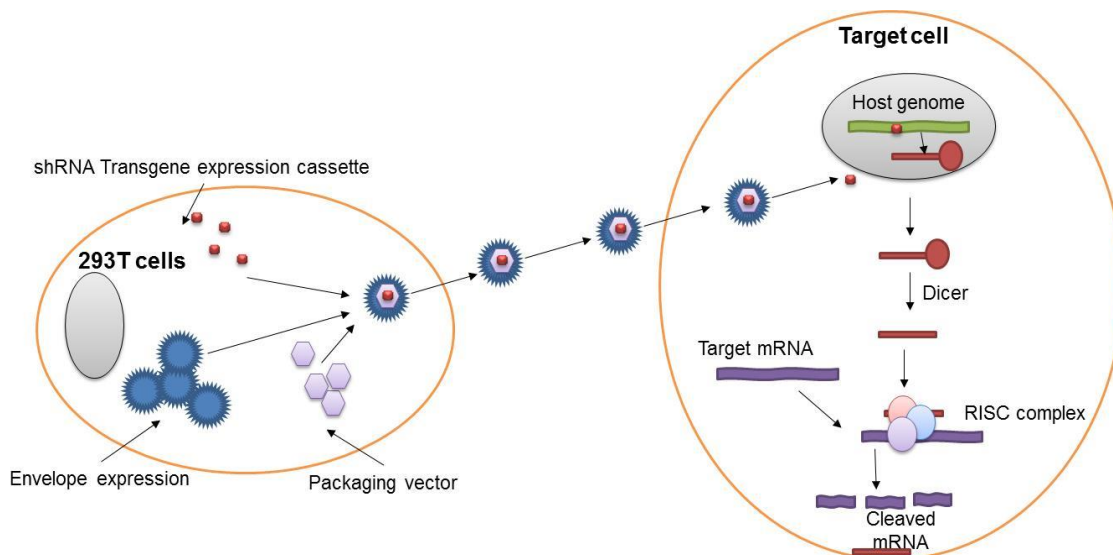
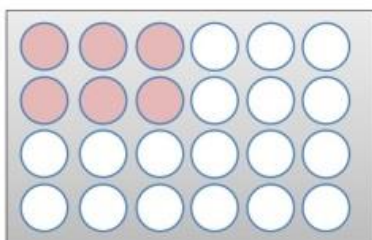


Figure 2.9: Schematic diagram of non-replicating lentiviral vector for stable shRNA expression

Suitable host cells (such as 293T cells) are transfected with a mixture of plasmids consisting of
i) an shRNA expression cassette
ii) a packaging cassette (psPAX2)
iii) a heterologous (VSV-G) viral envelope expression cassette (pMD2.G). The generated lentivirus is then used to transduce the desired cell type for shRNA expression. Because only the vector containing the shRNA expression cassette (devoid of the viral structural genes) integrates into the host cell genome in the transduced cells, shRNA is continually expressed but infectious virus is not produced. This figure is based on (Manjunath et al., 2009)

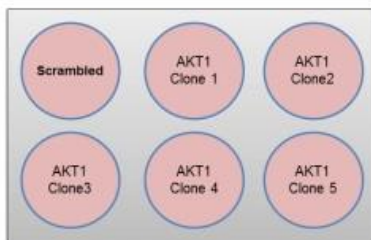
shRNA methodology

1. CLL cells plated out at 7×10^6 cells/ml in 24 well plate



2. Add virus particles to CLL cells and centrifuge at 1200g for 2 hours at RT.

3. Plate out 7×10^6 CLL cells which have had virus particles added in 2ml on CD40L fibroblasts.



4. Harvest cells 48-72 hours post plating out
5. Lyse cells, protein determination, run gel, western blot for AKT1.

Figure 2.10: Infection of CLL cells with lentiviral particles containing shRNAs specific to AKT1 or AKT2

Broadly, CLL cells were plated out at 7×10^6 cells/ml in 24 well plate, virus particles were added to CLL cells and centrifuged at 1200g for 2 hours at RT. Finally, 7×10^6 CLL cells which have had virus particles added were plated out in 2ml on 6-well CD154-expressing fibroblasts. 48-72 hours post plating out, CLL cells were harvested and GFP expression was checked using FL-1 on the flow cytometry, otherwise cells were lysed, protein determined and western blotted for expression of AKT1.

2.1.7 Western blotting analysis

Western blotting analysis allows for the detection and relative quantitation of the expression of specific proteins and phospho-proteins from a lysate extracted from cells (MacPhee, 2010). In broad terms there are three stages: firstly, cellular proteins are separated based on mass and charge by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) (Figure 2.11A). Secondly, proteins are transferred and immobilised onto a membrane (Figure 2.11B). Finally, proteins on the membrane are detected with either horseradish peroxidase (HRP) conjugated primary antibodies or primary antibodies followed by HRP conjugated secondary-antibodies, and the addition of enhanced chemiluminescence (ECL) substrates (Figure 2.11C) (Mahmood and Yang, 2012).

There are several factors worth considering when performing Western blotting analysis. Firstly, how cells or tissues are lysed. Protease and phosphatase inhibitors should always be included in lysis buffers to prevent protein degradation or modification. If protein-protein interactions are examined, a non-denaturing lysis buffer is preferable to minimise the disruption of these interactions (MacPhee, 2010).

Secondly, the SDS-PAGE. SDS is a detergent which denatures proteins by disrupting the hydrophobic regions resulting in them being negatively charged. Western blotting analysis uses two different types of gel: a stacking and a resolving gel. The stacking gel has low % acrylamide and is very porous so it separates the proteins poorly, but allows them to form a thin, sharp well-defined band before running on the separating gel, which separates the proteins by their mass. The % acrylamide in the resolving gel needs to be determined: if studying a high molecular mass protein, a low % gel is suggested, and conversely if studying a low molecular mass protein, a high % gel is suggested. High molecular mass proteins can be difficult to transfer, but there are modified protocols for such situations (Figure 2.11A) (MacPhee, 2010).

Thirdly, the transfer. Negatively charged proteins travel towards the positive electrode and transfer onto a membrane of either polyvinylidene difluoride (PVDF) or nitrocellulose. The proteins bind to the membrane non-covalently through hydrophobic interactions. Membranes have differing porosity and have limited capacity to bind protein, so it is prudent to consider how much protein lysate to run per lane on the gel. PVDF offers a higher binding capacity and is physically stronger

than nitrocellulose, but also has a higher background binding (Gibbons, 2014). Efficiency of transfers can be assessed by staining the membrane with Ponceau S (or other dyes) or by examining pre-stained molecular mass markers (Figure 2.11B) (MacPhee, 2010).

Finally, protein detection. Blocking a membrane blot serves to prevent non-specific binding of antibodies to the membrane as well as re-nature antigenic sites. Blocking buffers may contain either 5% non-fat dried milk powder or 1% bovine serum albumin (BSA). BSA is preferred when using phosphoprotein antibodies, since milk contains casein, which itself is a phosphoprotein. Washing steps are critical for ensuring low background in image acquisition. Choice of primary antibodies is important; monoclonal are generally more specific, however if the antigenic site is affected by denaturation, then polyclonal antibodies may better serve the purpose. Chemiluminescence is the most common detection method used in Western blotting analysis, where luminol is oxidised by hydrogen peroxide and light released during the chemical reaction is captured by the detector. In order to maintain light production, additional reagents are included in the reaction to enhance output of light. Signals can either be detected using ECL-rated film or Charge-coupled device (CCD) camera systems (MacPhee, 2010). Western blotting analysis is a semi-quantitative technique since detection of protein is not linear across all concentrations (Figure 2.11C) (Mahmood and Yang, 2012). Densitometry is often used to quantify the signal of the bands of interest on the blots. There are many other protein detection methods, including enzyme-linked assays, such as alkaline phosphatase linked antibodies and fluorescent labelling of proteins (Kurien and Scofield, 2006), which will not be discussed further here.

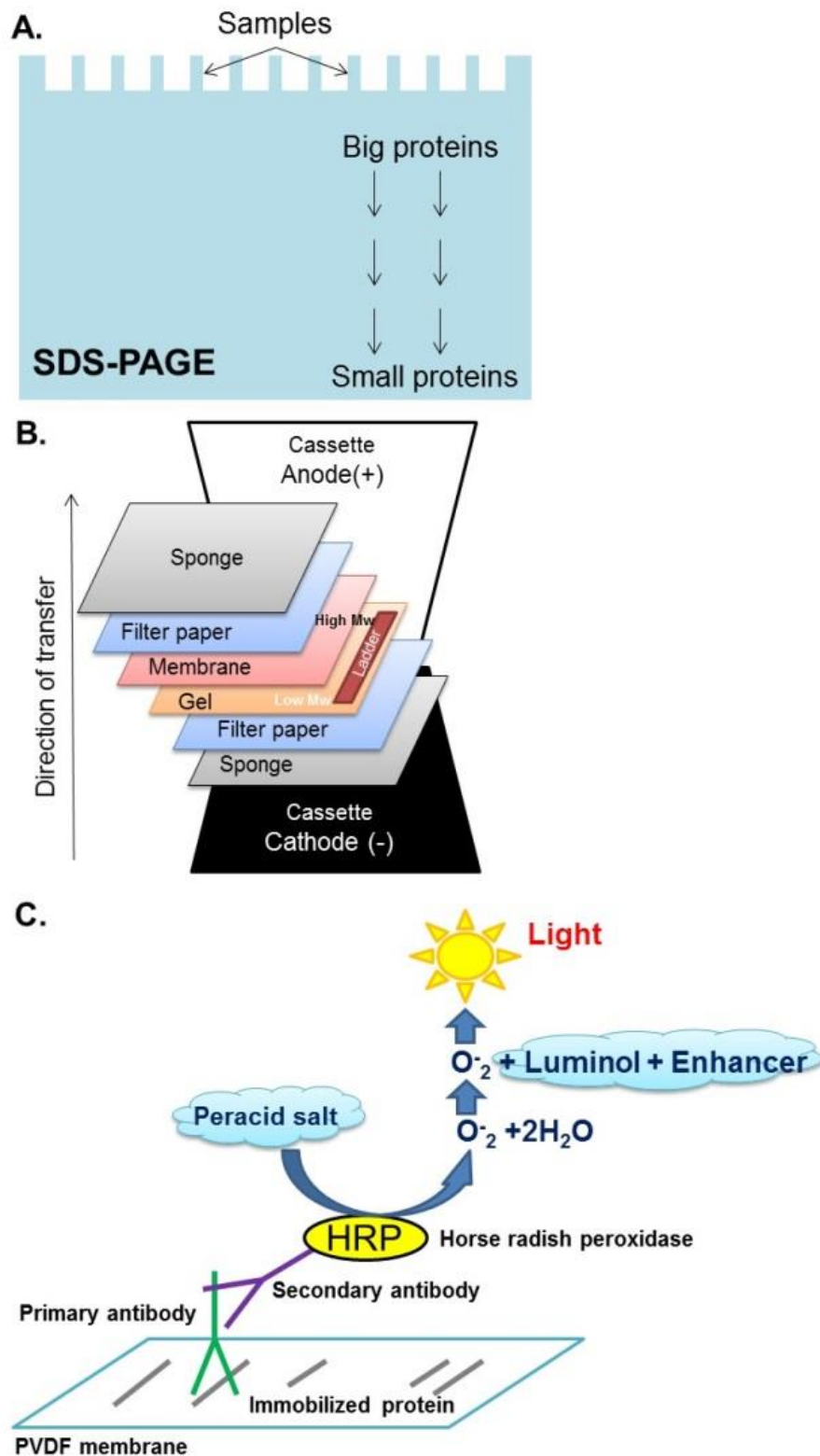


Figure 2.11: Western blotting

Western blotting allows for the detection of proteins and phospho-proteins.

A. Firstly, proteins are separated by mass and charge by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS).

B. Proteins are then transferred and immobilised onto a PVDF membrane

C. The PVDF membrane is then probed with primary antibody, followed by a HRP conjugated secondary-antibody. ECL is applied and signal is then detected using a CCD camera.

2.1.7.1 Cell lysis

Before lysis, CLL cells were collected in 1.5ml tubes and washed once in ice-cold PBS. Approximately 4×10^6 cells were lysed in 100 μ l of RIPA buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1% (w/v) Triton-X100, 0.1% (w/v) SDS, 0.5% (w/v) Na Deoxycholate supplemented with 1 in 100 dilution of protease and phosphatase inhibitor cocktails. Protease inhibitor cocktail (#P8340, Sigma) and phosphatase inhibitor cocktail (#524625, Millipore) were obtained commercially. Cells were vortexed and left on ice for 10 mins. To complete lysis of cells, samples were then sonicated 7 x (30s ON/ 30s OFF) on “high setting” in the Diagenode Bioruptor® Standard sonication device (Seraing, Belgium). After sonication, cell lysates were clarified by centrifuging at 10,000g for 20 min at 4°C and supernatant transferred into a new tube.

Alternatively, approximately 4×10^6 cells were lysed in 100 μ l of break-up buffer (10mM Tris-HCl, pH7.4, 5mM MgCl₂, 1% Triton X-100, 0.1M NaCl) supplemented with 1 in 100 dilution of protease and phosphatase inhibitor cocktails and were pulse sonicated manually.

2.1.7.2 Protein concentration determination

Protein concentration was determined using the Bio-Rad DC™ (detergent compatible) protein assay kit (#500-0116, Bio-Rad laboratories, Hertfordshire, UK). This is a colorimetric assay for protein concentration following detergent solubilisation. For more detailed protocol, see Appendix 1.6 Protein concentration determination. The Bio-Rad DC™ protein assay is an adaptation of the Lowry assay, which relies on two reaction steps resulting in colour change. Firstly, protein reacts with copper in an alkaline medium, then the copper-bound protein can reduce Folin reagent by loss of oxygen atoms, thereby producing reduced species which have a blue colour (Lowry et al., 1951, Peterson, 1979).

2.1.7.3 SDS-PAGE and immunoblotting

Cell lysates along with a BenchMark™ Pre-Stained Protein Ladder (Life Technologies Catalog No: 10748-010) were boiled at 95°C for 5 min in 4 x Laemmli buffer (250 mM Tris-HCl (pH 6.8), 8% SDS, 40% Glycerol, 0.02% Bromophenol blue, 10% β -mercaptoethanol) or 2 x Sample buffer (125mM Tris-HCl, pH 6.8, 30%

Glycerol, 4% SDS, 0.1% Bromophenol Blue, 20 mM EDTA, 5% β -mercaptoethanol) and were loaded and separated on an Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Immobilon-P Membrane, PVDF, 0.45 μ m, 26.5cm x3.75m roll, Catalogue Number : IPVH00010, Millipore Corporation, Bedford, MA). PVDF membranes were probed with the appropriate primary antibodies in either 3% BSA/TBST (if a phospho-specific antibody) or 5% milk/TBST overnight at 4 °C (Table 2.7).

2.1.7.4 Image acquisition

Immunoreactivity was detected with the relevant horseradish peroxidase-labelled secondary antibodies (Table 2.8: Secondary antibodies) which, in turn, were visualized on an Image Reader LAS-1000 (Fujifilm, Tokyo, Japan) using either Immobilon Western Chemiluminescent HRP Substrate (WBKLS0100, Millipore) or ECL ADVANCE Western blotting Detection Kit (25190014, GZRPN2135 - GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.1.7.5 Densitometry

For quantification of the data, the images were further analysed on the same instrument using 2D Densitometry Aida Image Analyzer software (Fujifilm).

In selected experiments, membranes were re-probed after stripping in buffer containing 62.5 mM Tris-HCl (pH 6.7), 2% SDS and 100 mM 2-mercaptoethanol at 50°C for 30 min.

2.1.8 Flow cytometry

Flow cytometry measures several parameters of cells in liquid suspensions simultaneously, as they pass through a beam of light. Cells or particles between 0.2-50 μm are suitable for analysis by most flow cytometers. Although flow cytometry is very useful and efficient in analysing cells of the haematological system, its major disadvantage is that the cells to be analysed must be in liquid suspensions. If solid tumours were to be studied, cells would have to be disaggregated before being analysed in this manner.

Flow cytometers can be used to measure relative size, internal complexity and fluorescence of the cells or particles of interest (Ormerod, 2000b). Figure 2.12 displays an optical configuration of a flow cytometer with four channels (FL1-4); up to four fluorochemicals can be used at a time, thus maximising the information gained from limited amounts of sample in a cost-effective manner.

Flow cytometry is most commonly used for immunophenotyping using fluorescently-labelled monoclonal antibodies against surface markers to identify subsets of cells of interest (Craig and Foon, 2008, Calvo et al., 2011). Flow cytometry can also be used to perform DNA analysis revealing cell distribution within the major phases of the cell cycle (Darzynkiewicz and Huang, 2004) or estimating the frequency of apoptotic cells (Darzynkiewicz et al., 1992). It can also be used to analyse multiple cytokine, chemokine, immunoglobulin, or cell signalling targets from a single sample, using a multiplexed assay such as the one from BDTM Cytometric Bead Array (CBA) (Varro et al., 2007). In recent years, flow cytometry has been coupled with cell sorting modules, to effectively identify and isolate subsets of cell populations from a single sample, for further experimentation such as genetic sequencing, RNA profiling and proteomics (Ibrahim and van den Engh, 2007).

The BD Fluorescence-activated cell sorting (FACS) CaliburTM was used for all experiments in this thesis.

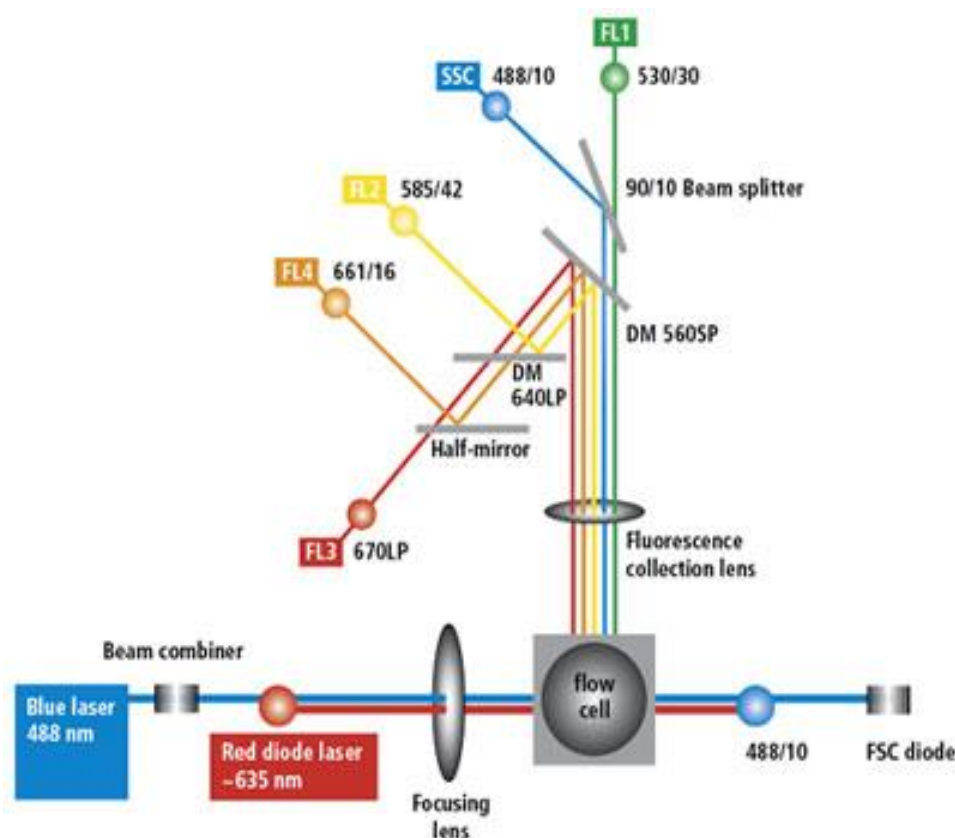


Figure 2.12: Optical configuration of a flow cytometer

The standard BD FACS Calibur system includes a 15-mW, 488-nm, argon-ion laser and detectors for three or four fluorescent parameters. The FL4 option provides a second 635-nm, red-diode laser and an additional detector. Laser light is focused onto the flow cell by a focusing lens. As fluorescently tagged particles intercept the laser light in the flow cell, scattered and fluorescent light provide information about particle size, shape, granularity, and fluorescence intensity. Forward scatter (FSC) signal is collected by the FSC diode. Side scatter (SSC) and fluorescent signals are collected by a fluorescence collection lens and spectrally split by a collection of dichroic mirrors (DMs) and filters. Figure image and legend taken from <https://www.bdbiosciences.com/in/instruments/facscalibur/features/index.jsp> https://www.bdbiosciences.com/documents/BD_FACSCalibur_instructions.pdf respectively.

2.1.8.1 Analysis of expression of CD154 in transfected fibroblasts by FACS

The expression of CD154 was routinely checked by FACS using Cell Dissociation Solution (#C5789, Sigma) to dissociate the cells and incubating the fibroblasts with FITC mouse anti-human CD154 (Cat no. 555699, BD Biosciences) or FITC mouse IgG1 κ isotype control antibody (Cat no. 555748, BD Biosciences).

Fibroblast monolayers were removed from culture dishes by washing twice with pre-warmed PBS and then adding an appropriate volume of Cell Dissociation Solution (Cat no. C5789, Sigma) to the monolayer. Once the fibroblasts had detached from the plate (as observed under the microscope), cells were collected and spun at 200g for 5min, washed with media, spun again and finally resuspended in PBS.

5 x 10⁵ cells were aliquotted into each FACS tube containing 0.5 ml of PBS/0.1% BSA and incubated for 10 minutes in the dark with either 10 µl of FITC mouse anti-human CD154 (BD Biosciences, cat. no. 555699) or 10 µl FITC mouse IgG1 κ isotype control antibody (BD Biosciences, cat. no. 555748). FACS tubes containing the cells were spun at 200g for 5 min and resuspended in 0.5 ml of PBS/0.1% BSA. CD154 expression on the fibroblasts was measured by FACS on FL-1 on the BD FACS Calibur™.

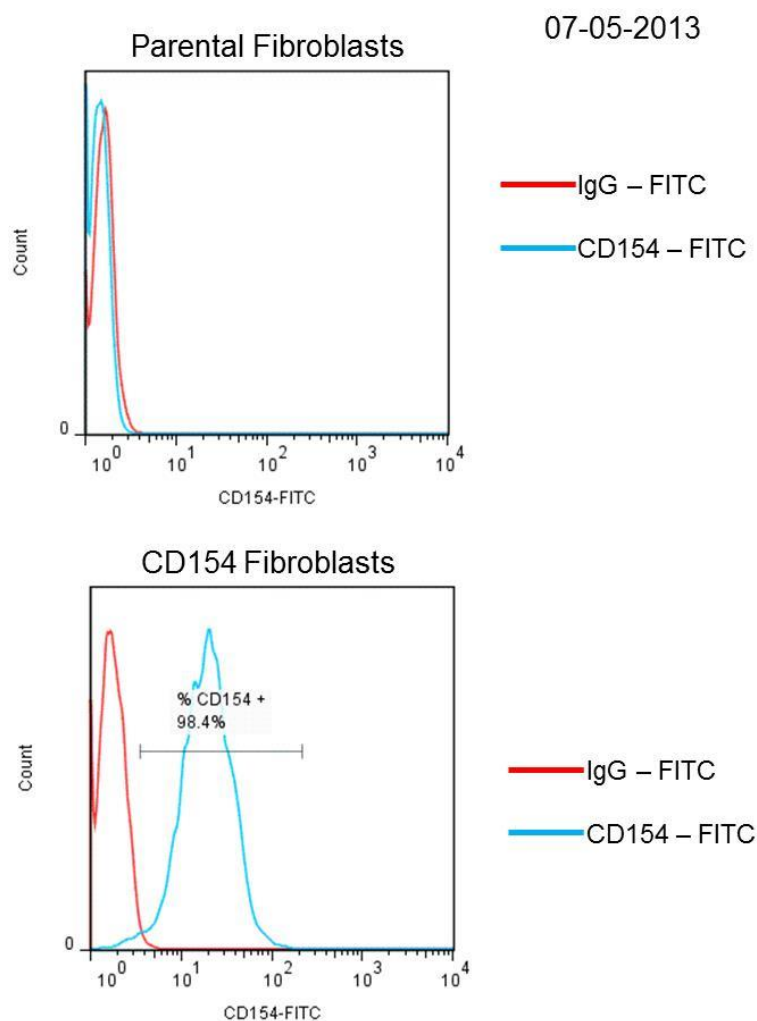


Figure 2.13: Analysis of CD154 in transfected fibroblasts by FACS

Fibroblast monolayers were removed from culture dishes by washing twice with pre-warmed PBS and then adding an appropriate volume of Cell Dissociation Solution (#C5789, SIGMA) to the monolayer. Once the fibroblasts had detached from the plate (as observed under the microscope), cells were collected and spun at 200g for 5min, washed with media, spun again and finally resuspended in PBS. 5 x 10⁵ cells were aliquotted into each FACS tube containing 0.5 ml of PBS/0.1% BSA and incubated for 10 minutes in the dark with either 10 µl of FITC mouse anti-human CD154 (BD Biosciences, cat. no. 555699) or 10 µl FITC mouse IgG1 κ isotype control antibody (BD Biosciences, cat. no. 555748). FACS tubes containing the cells were spun at 200g for 5min and resuspended in 0.5 ml of PBS/0.1% BSA. CD154 expression on the fibroblasts was measured by FACS on FL-1 on the BD FACSCalibur™.

2.1.8.2 CFSE staining

CFSE staining of CLL cells was monitored using FL-1 on FACS Calibur™. Data was analysed using FlowJo 7.6.5 (2.3.3 Flow Jo) and the percentage of divided cells was calculated accordingly.

2.1.8.3 Flow cytometry analysis of cell viability using propidium iodide

CLL cells were incubated in the presence or absence of indicated reagents at a density of 5×10^6 cells/ml in multi-well plates. At the end of the treatment period, drug-induced killing was determined by using a flow cytometry method employing propidium iodide (Figure 2.14a). Briefly, equal volumes of cells in media to propidium iodide (100 µg/ml in PBS) solution (P4864-10ML, Sigma-Aldrich) were mixed in a FACS tube and incubated on ice in the dark for 10-15 minutes. Analysis was carried out using a FACS Calibur flow cytometer. Live cells appear as PI-dim, while dead cells are PI-bright. 10,000 events were collected as standard.

The formula $[(\text{Viability}_{(UT)} - \text{Viability}_{(T)})/\text{Viability}_{(UT)}] \times 100$ was used to define percentage killing relative to untreated (UT) controls.

Work performed previously in the laboratory showed that results were comparable irrespective of whether cell death was measured by single-staining with PI or double-staining with annexin V and PI [Supplementary Figure 1b from (Melarangi et al., 2012)].

There is however, a downside of only using PI. Firstly, PI naturally enters cells with time. Secondly, PI only measures late cell death. In order to measure early events in apoptosis, the addition of Annexin V (a Ca^{2+} dependent phospholipid-binding protein with high affinity for PS), can be used to measure the presence of phosphatidylserine (PS) on the outer surface of the plasma membrane which becomes exposed in the early stages of apoptosis. PI alone is therefore not a measure of apoptosis, it is only a measure of cell death (Figure 2.15) (Vermes et al., 1995, Vermes et al., 2000).

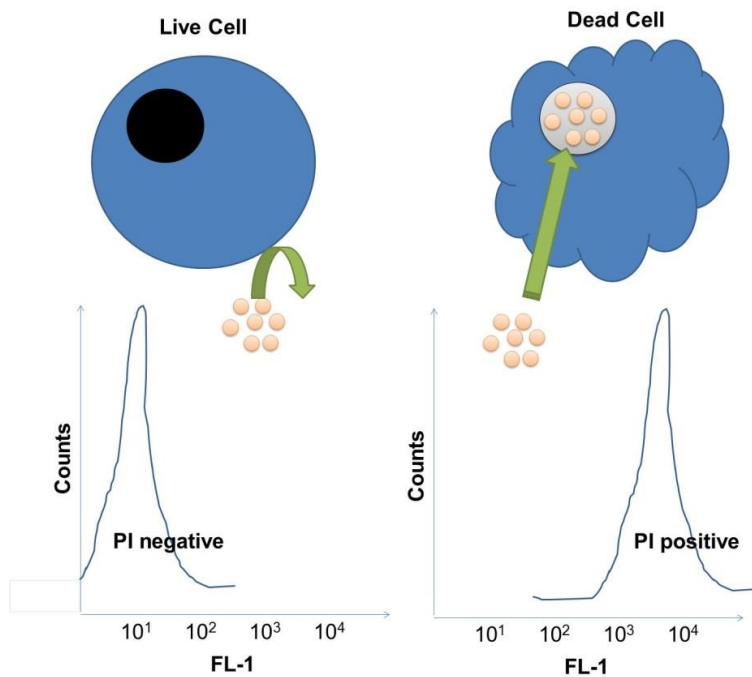


Figure 2.14: Propidium iodide can freely enter dead cells, but is selectively excluded from live cells.

Propidium iodide (PI) is a standard viability dye in flow cytometry. Viable cells with intact membranes exclude PI whereas membranes of dead and damaged cells are permeable to PI. PI binds to double stranded DNA by intercalating between base pairs. When PI is bound to nucleic acids, the fluorescence excitation maximum is 535 nm and the emission maximum is 617 nm (Darzynkiewicz et al., 1992).

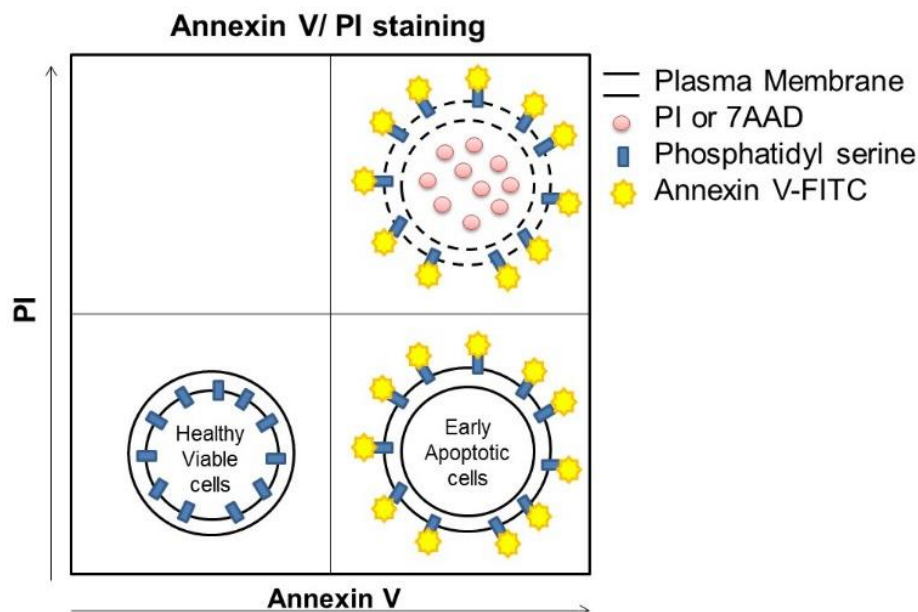


Figure 2.15: Flow cytometry data of dual staining with with annexin V and PI.

In a live, intact cell, PI cannot enter the cell and label dsDNA/RNA and phosphatidyl serine (PS) is maintained exclusively on the inner leaflet, resulting in negative staining for both dyes. Annexin V labels externalized PS- on the cell surface following the initiation of apoptosis. During the latter stages of apoptosis both PS is exposed and PI can enter the cell due to the loss of membrane integrity.

2.2 Materials

2.2.1 Antibodies

The details of all primary antibodies used for Western blotting analysis are provided in Table 2.7 and details of related secondary antibodies in Table 2.8. Table 2.9 provides details of antibodies used for FACS analysis.

Table 2.7: Primary antibodies

Description	Company	Catalogue number	Source	Dilution	Molecular Weight (kDa)
β -Actin (AC-74 clone)	SIGMA	A5316	Mouse mAb	1:20k	42
PI3K p110 isoforms					
PI3K p110 α (C73F8)	CST	#4249	Rabbit mAb	1:1000	110
PI3K p110 β (C33D4)	CST	#3011	Rabbit mAb	1:1000	110
PI3K p110 δ	Abcam	ab1678	Rabbit polyclonal	1:1000	110
PI3K p110 γ (D55D5)	CST	#5405	Rabbit mAb	1:1000	110
AKT					
Akt	CST	#9272	Rabbit mAb	1:1000	60
p-Akt (Ser473) (D9E) XP®	CST	#4060	Rabbit mAb	1:1000	60
Phospho-Akt (Thr308) (C31E5E)	CST	#2965	Rabbit mAb	1:1000	60
AKT substrates					
Phospho-GSK-3 β (Ser9) XP® (D85E12)	CST	#5558	Rabbit mAb	1:1000	46
GSK-3 β (3D10)	CST	#9832	Mouse mAb	1:1000	46
p-GSK-3 α/β (Ser21/9) (D17D2)	CST	#8566	Rabbit mAb	1:1000	46 GSK-3 β 51 GSK-3 α
GSK3 Alpha/Beta (21A)	LT	44-610	Mouse mAb	1:1000	46 GSK-3 β 51 GSK-3 α
p-MDM2 (Ser166)	CST	#3521	Rabbit mAb	1:1000	90
MDM2 (SMP14) HRP	SC	sc-965-HRP	None	1:500	90
p-PRAS40 (Thr 246) (C77D7)	CST	#2997	Rabbit mAb	1:1000	40
PRAS40 (D23C7) XP®	CST	#2691	Rabbit mAb IgG	1:1000	40
Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP®	CST	#4858	Rabbit mAb	1:1000	32
S6 Ribosomal Protein (5G10)	CST	#2217	Rabbit mAb	1:1000	32
Phospho-4E-BP1 (Ser65) Antibody	CST	#9451	Rabbit mAb	1:1000	15-20
Phospho-4E-BP1 (Thr37/46) (236B4)	CST	#2855	Rabbit mAb	1:1000	15-20
4E-BP1 (53H11)	CST	#9644	Rabbit mAb	1:1000	15-20
CDK Antibody Sampler Kit #9868					
cdc2 (POH1)	CST	#9116	Mouse mAb	1:1000	34
CDK2 (78B2)	CST	#2546	Rabbit mAb IgG	1:1000	33
CDK4 (D9G3E)	CST	#12790	Rabbit mAb	1:1000	30
CDK6 (D4S8S)	CST	#13331	Rabbit mAb	1:1000	36
Cyclin Antibody Sampler Kit #9869					
Cyclin A2 (BF683)	CST	#4656	Mouse mAb (IgE)	1:1000	55

Cyclin D2 (D52F9)	CST	#3741	Rabbit mAb (IgG)	1:1000	31
Cyclin D3 (DCS22)	CST	#2936	Mouse mAb (IgG1)	1:1000	31
Cyclin E1 (HE12)	CST	#4129	Mouse mAb (IgG1)	1:1000	48-56
CDKis					
p21 Waf1/Cip1 (DCS60)	CST	#2946	Mouse mAb	1:2000	21
p27 Kip1 (D69C12) XP®	CST	#3686	Rabbit mAb	1:1000	27
STAT antibodies					
p-STAT3 (Tyr 705) (3E2)	CST	#9138	Mouse mAb	1:1000	79 STAT3β 86 STAT3α
p-STAT6 (Tyr641)(C11A12)	CST	#9364	Rabbit mAb	1:1000	100
STAT3 (C-20)	SC	sc-482	Rabbit polyclonal IgG	1:200	79 STAT3β 86 STAT3α
STAT6 (M-20)	SC	sc-981	Rabbit polyclonal IgG	1:200	119
Akt Isoform Antibody Sampler Kit #9940					
AKT1 (C73H10)	CST	#2938	Rabbit mAb (IgG)	1:1000	60
AKT2 (D6G4)	CST	#3063	Rabbit mAb (IgG)	1:1000	60
AKT3(L47B1)	CST	#8018	Mouse mAb (IgG1)	1:1000	60
AKT (pan) (C67E7)	CST	#4691	Rabbit mAb (IgG)	1:1000	60
Others					
GFP antibody (B-2)	SC	sc-9996	Mouse mAb	1:200	27
Bcl-XL (54H6)	CST	#2746	Rabbit mAb	1:1000	45
PARP (C2-10)	R&D	4338-MC-50	Mouse mAb (IgG1)	1:1000	118 = 89 +24

CST – Cell signalling technologies, UK supplier: New England Biolabs, Inc. (NEB).

SC – Santa Cruz, UK supplier: Insight Biotechnology Ltd.

LT - Life Technologies Ltd (previously Invitrogen)

R&D - R&D Systems, Minneapolis, MN

Table 2.8: Secondary antibodies

	Company	Description	Catalogue number	Dilution used
Mouse-HRP	Santa Cruz	goat anti-mouse IgG-HRP	sc-2055	1: 5000
Rabbit-HRP	Santa Cruz	goat anti-rabbit IgG-HRP	sc-2004	1: 5000

Table 2.9: Antibodies used for flow cytometry

	Company	Description	Cat #
IgG-FITC	BD Pharmingen™	FITC mouse IgG1 κ isotype control	555748
CD154-FITC	BD Biosciences	FITC mouse anti-human CD154	555699
CD5-FITC	BD Biosciences	L17F12	345781
CD19-FITC	BD Biosciences	SJ25C1	340409
IgG-FITC	BD Biosciences	Mouse IgG1, κ (x40)	345815
CD5-PE	BD Biosciences	(Leu-1)	347307
CD19-PE	BD Biosciences	4G7	345777
IgG-PE	BD Biosciences	Mouse IgG1, κ (x40)	345816
CD19-PerCP	BD Biosciences	CD19-PerCP Cy5.5 (SJ25C1)	332780
IgG- PerCP	BD Pharmingen™	CD19- PerCP-Cy™5.5 Mouse IgG1, κ	552834

2.2.2 Reagents / drugs

2.2.2.1 Bendamustine

Bendamustine hydrochloride hydrate (Cat. no. B5437, Sigma) was made up to 100 mM in DMSO and aliquotted in 5 μ l aliquots (Figure 2.16).

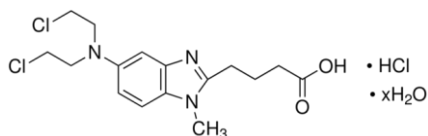


Figure 2.16: Bendamustine hydrochloride hydrate

2.2.2.2 Dexamethasone

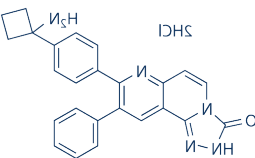
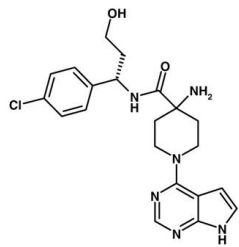
Dexamethasone (100 mg, Dexamethasone-Water Soluble suitable for cell culture, BioReagent, Cat no. D2915, Sigma) was made up to 200 μ M in water and aliquotted in 5 μ l aliquots.

2.2.2.3 AKT inhibitors

AZD5363 was provided by Astra Zeneca, Alderley Park, Cheshire, and was made up to 100 mM in DMSO and aliquotted in 10 µl aliquots.

MK-2206 2HCl catalog no. S1078, was purchased from Selleck Chemical, Stratech Scientific Ltd., Suffolk, and was made up to 20 mM in DMSO and aliquotted in 10 µl aliquots.

Table 2.10: AKT inhibitors.

	MK-2206 2HCl		AZD5363
			
	(Yan, 2009, Hirai et al., 2010)		(Davies et al., 2012)
Targets	IC ₅₀ (nM)	Targets	IC ₅₀ (nM)
Akt1	8	Akt1	3
Akt2	12	Akt2	7
Akt3	65	Akt3	7
	No inhibitory activities against 250 other protein kinases observed.	P70S6K	6
		PKA	7
		ROCK1	470
		ROCK2	60
	MK-2206 by itself causes growth inhibition of the eight cell lines with an IC ₅₀ that ranges between 3.4 - 28.6 µM		Inhibition of AKT substrates in cells µM
		pGSK3β	0.76 (BT474c; Western)
			0.06 (LNCaP; Western)
			0.38 (MDA-MB-468; Acumen)
		pPRAS40	0.31 (BT474c; Western)
			0.22 (LNCaP; Western)
			0.39 (MDA-MB-468; Acumen)
		pFOXO3a translocation	0.69 (BT474c)
Clinical trial	Phase 2	Clinical trial	Phase 2

2.2.2.4 PI3K inhibitors.

Owing to the different isoforms of PI3K p110 catalytic subunit, there are many PI3K inhibitors available, some having preferences for specific isoforms (Table 2.11: PI3K inhibitors.).

5 mg of LY294002, a pan-PI3K inhibitor (Cat No. 440202) was purchased from Calbiochem, San Diego, United States, and was made up to 10 mM aliquots in DMSO. LY294002 is a cell-permeable, potent, reversible and specific phosphatidylinositol 3-kinase inhibitor ($IC_{50} = 1.3 \mu M$) that acts on the ATP-binding site of the enzyme (Vlahos et al., 1994). LY294002 is the first synthetic molecule known to inhibit PI3K $\alpha/\delta/\beta$ with IC_{50} of $0.5 \mu M/0.57 \mu M/0.97 \mu M$, respectively (Chaussade et al., 2007). LY294002 also inhibits DNA-PKc, but does not affect the activities of EGF receptor kinase, MAP kinase, PKC, PI 4-kinase, S6 kinase, or c-Src even at $50 \mu M$.

Pictilisib (GDC-0941), PI3K α/δ inhibitor cat. no. S1065; Idelalisib (CAL-101, GS-1101) PI3K δ inhibitor cat. no. S2226; and Duvelisib (IPI-145, INK1197), PI3K δ/γ inhibitor cat. no. S7028 were purchased from Selleck Chemical, Stratech Scientific Ltd, Suffolk.

Table 2.11: PI3K inhibitors.

	GDC-0941			CAL-101 (Idelalisib, GS-1101)			Duvelisib (IPI-145, INK1197)	
	PI3K α / δ inhibitor			PI3K δ inhibitor			PI3K δ / γ inhibitor	
<i>In vitro</i> kinase assay references	(Folkes et al., 2008)			(Lannutti et al., 2011)			(Winkler et al., 2013)	
Target		Value	Target		Value	Target		Value
p110α	IC ₅₀	3 nM	p110α	IC ₅₀	820 nM	PI3Kα	IC ₅₀ (3mM ATP)	1602 nM
p110β	IC ₅₀	33 nM	p110β	IC ₅₀	565 nM	PI3Kβ	IC ₅₀ (3mM ATP)	85 nM
p110δ	IC ₅₀	3 nM	p110δ	IC ₅₀	2.5 nM	PI3Kδ	IC ₅₀ (3mM ATP)	2.5 nM
p110γ	IC ₅₀	75 nM	p110γ	IC ₅₀	89 nM	PI3Kγ	IC ₅₀ (3mM ATP)	27 nM
PI3Kα	Ki					PI3Kα	Ki	25900 pM
PI3Kβ	Ki					PI3Kβ	Ki	1564 pM
PI3Kδ	Ki					PI3Kδ	Ki	23 pM
PI3Kγ	Ki					PI3Kγ	Ki	243 pM
mTOR	IC ₅₀		mTOR	IC ₅₀	>1 μ M			
mTOR	Ki app	0.58 μ M						
C2β	IC ₅₀	0.67 μ M	C2β	IC ₅₀	>1 μ M			
DNA-PK	IC ₅₀	1.23 μ M	DNA-PK	IC ₅₀	6.729 μ M			
Vps34	IC ₅₀	>10 μ M	hVps34	IC ₅₀	978 nM			
			PIP5Kα	IC ₅₀	>1 μ M			
			PIP5Kβ	IC ₅₀	>1 μ M			
Cell based work	(Junttila et al., 2009)			(Herman et al., 2010)				

2.2.3 Recombinant proteins

Recombinant proteins p110 $\alpha, \beta, \delta, \gamma$ were provided by AstraZeneca (Alderley Park, Cheshire, UK). GFP recombinant protein (Recombinant EGFP, STA-201, 100 μ g) was purchased from Cell Biolabs, Inc. (San Diego, CA, USA) and distributed by CAMBRIDGE BIOSCIENCE LTD (Cambridge, UK).

2.3 Statistical analysis

IBM® SPSS® Statistics Version 21, Release 21.0.0.1. 64-bit edition and Microsoft Office Excel 2010 were used to calculate the statistics in this project.

2.3.1 Parametric statistics: Paired student t-test

Parametric statistics assumes that the data are continuous and normally distributed. Two-tailed, paired student t-test was used and *p* values calculated using Microsoft Office Excel 2010.

2.3.2 Non-parametric statistics: Mann-Whitney U and Kruskal-Wallis

Non-parametric statistics make no assumptions about the probability distributions of the variables being assessed. IBM® SPSS® Statistics Version 21, Release 21.0.0.1. 64-bit edition was used to perform Mann-Whitney U (2 distinct groups) and Kruskal-Wallis tests (1-way ANOVA, *k* samples, it extends the Mann-Whitney U test when there are more than two groups).

2.3.3 Flow Jo software

FlowJo software (7.6.5 version) was used to analyse proliferation data of CFSE-labelled CLL cells.

Chapter 3 : The role of AKT in CLL-cell survival

3.1 Background and aims

Whilst there have been no reports of activating mutations in *PIK3CA*, *PIK3CD*, *PIK3CG*, *PTEN* and *PIK3R1* and *AKTE17K* in CLL (Marincevic et al., 2009, Brown et al., 2012, Mahmoud et al., 2008, Zenz et al., 2008a), AKT certainly seems to be important for CLL-cell survival, especially in mediating survival signals in response to stimuli from the microenvironment.

Whether AKT is constitutively active (as measured by phosphorylation on serine 473 or threonine 308) in CLL cells has been a matter for debate. Some groups were able to detect phosphorylated AKT in unstimulated CLL cells (Plate, 2004, Zhuang et al., 2010, de Frias et al., 2009). For instance, Barragan and colleagues found detectable phosphorylated AKT in seven out of ten CLL samples (Barragan et al., 2006) and Zhuang and colleagues could detect phosphorylated AKT in almost all CLL samples (Zhuang et al., 2010). However, others found little or no phosphorylated AKT in CLL cells (Ringshausen et al., 2005, Muzio et al., 2008). These differences may be accounted for by the following factors: firstly, the apparent heterogeneity of CLL as a disease; secondly, the varied amount of protein loaded in individual experiments; thirdly the different antibodies used, and finally, different methods of extraction used in the studies.

Nevertheless, AKT has been shown to be involved in the survival of CLL cells in response to BCR stimulation (Bernal et al., 2001, Petlickovski et al., 2005, Longo et al., 2008). Also, treatment of CLL cells with siRNA to AKT1 or AKT inhibitors (A-443654 and AKTi-1/2) decreased levels of p-GSK3 α and MCL1 protein and resulted in CLL-cell apoptosis (Zhuang et al., 2010, de Frias et al., 2009).

AKT has been shown to be activated in CLL cells by a variety of stimuli, many of which are relevant to the CLL microenvironment. For example, AKT is activated by albumin in plasma (Jones et al., 2003, Wickremasinghe et al., 2001) and activated in CLL cells upon co-culture with murine marrow stromal cells or HS-5 stromal cells (Hofbauer et al., 2010, Edelmann et al., 2008). AKT is also activated in CLL cells by CXCL12 or BAFF, which signals via CXCR4, and BAFF-R on CLL cells, respectively (Niedermeier et al., 2009, Nishio et al., 2005, Rickert et al., 2011). AKT

is activated upon CLL-cell adhesion to endothelial cells and integrin CD49d (Fiorcari et al., 2013). CD40 stimulation (with anti-CD40 monoclonal antibodies or soluble CD40L) also promotes CLL-cell survival through the PI3K-AKT pathway (Bernal et al., 2001, Cuni et al., 2004, Herman et al., 2010).

Despite its importance in mediating survival signals in the CLL microenvironment, the effect of targeting AKT with chemical inhibitors in CLL cells cultured under conditions that model the microenvironment has not been well characterised.

Hofbauer and colleagues have shown that treatment with an inhibitor of AKT, AIX, in CLL cells co-cultured with marrow stromal cells resulted in inhibition of AKT activation and induction of apoptosis of CLL cells (Hofbauer et al., 2010). Since the CLL microenvironment (lymph nodes and bone marrow) are niches where CLL cells survive and are protected from the cytotoxic effects of chemotherapeutic agents, and proliferate, it is very important to understand the molecular signalling mechanisms mediating survival and proliferation so that CLL cells in these protective environments can be pharmacologically targeted.

One such mechanism involves the interaction between CLL cells and T cells in the lymph node through the CD40-CD154 signalling pathway. CD40-stimulation using *in vitro* models where CLL cells are stimulated with membrane-bound CD40L (mCD154) both promotes cell survival and prevents drug-induced apoptosis. CD40 stimulation of CLL cells results in increased expression of anti-apoptotic proteins such as BCLXL, A20, BFL1, survivin and cFLIP (Elgueta et al., 2009, Rickert et al., 2011, Vogler et al., 2009a, Zhuang et al., 2014), and hence resistance to chemotherapeutic agents such as fludarabine (Romano et al., 1998).

CD40 stimulation promotes CLL-cell survival through the activation of the NFκB (Schattner, 2000, Furman et al., 2000) and PI3K-AKT pathways (Bernal et al., 2001, Cuni et al., 2004, Herman et al., 2010). One report has suggested that activation of the AKT signalling pathway was dependent upon CD40 lipid raft association and independent of any signalling events (Nadiri et al., 2011). Irrespective of its mode of activation, AKT appears to be activated upon CD40 stimulation and thus targeting AKT in this setting may have therapeutic potential in CLL.

The PI3K inhibitors idelalisib and duvelisib have shown promising clinical activity in CLL and, given that AKT is an important downstream kinase of PI3K, it is speculated that targeting AKT may have similar effects.

In this part of the study, I thus sought to establish whether AKT is required for CLL-cell survival, in particular under conditions that mimic the protective tissue microenvironment. As AKT has been shown to be activated in CLL cells upon CD40 stimulation by soluble CD40 ligand or anti-CD40 antibodies (Cuni et al., 2004, Herman et al., 2010), I wished to confirm whether AKT was activated in CLL cells co-cultured with CD154-expressing fibroblasts. I also sought to establish if inhibition of AKT can sensitise CD40-stimulated CLL cells to killing by current chemotherapeutic agents (e.g. bendamustine), and if so, what the underlying mechanism is.

To measure AKT activation upon CD40 stimulation, I examined the phosphorylation status of AKT in CLL cells co-cultured with human CD154-expressing mouse fibroblasts. I then investigated the effect of AKT inhibition in the co-cultured CLL cells using a novel, selective inhibitor of AKT, AZD5363s, either alone or in combination with current chemotherapeutic drugs (e.g. bendamustine).

3.2 The effect of AKT inhibitor AZD5363 on the viability of CLL cells cultured under standard conditions.

3.2.1 AZD5363 inhibits AKT in CLL cells

Having established that AZD5363 is active in inhibiting AKT activity in Jurkat cells (see [Appendix 2.1](#) for details), I next sought to determine whether AZD5363 is also active in primary CLL cells. Initially, CLL cells (from sample #2814) were cultured for 24 h under standard conditions in the presence of AZD5363 at a range of concentrations. At the end of incubation, CLL cells were harvested for Western blotting analysis to examine the phosphorylation status of some well-characterised AKT substrates. Jurkat cells which had been incubated for 24 h with AZD5363 under standard conditions were also used for comparison. I probed for p-GSK3 α/β , p-MDM2, p-PRAS40, p-S6 and p-4E-BP1. As expected, AZD5363 induced a concentration-dependent decrease in phosphorylated GSK3 α/β , MDM2, PRAS40, S6 and 4E-BP1 in Jurkat cells (Figure 3.1A, right panels). CLL cells cultured under standard conditions for 24 h expressed detectable levels of phosphorylated GSK3 α/β , MDM2, PRAS40 and S6 and 4E-BP1. Treatment with AZD5363 resulted in a concentration-dependent de-phosphorylation of all of these AKT targets, indicating that the drug was active intracellularly in inhibiting AKT (Figure 3.1A, left panels). Since detectable levels of p-PRAS40 and p-S6 were very low in CLL samples when 8.8 μ g of CLL lysate was loaded in each lane (Figure 3.1A), I re-ran the Western blotting analysis by loading almost twice the amount of lysates (i.e. 15 μ g/lane). This time the concentration-dependent decrease in levels of p-PRAS40 was visible between 0-1 μ M AZD5363 and p-S6 visible between 0-3 μ M AZD5363 (Figure 3.1B). Densitometric analysis of signals corresponding to phosphorylated proteins relative to β -actin in CLL cells (sample #2814) indicated that the concentration that was required to achieve 50% inhibition of phosphorylation (often referred to as IC₅₀) for the following substrates was between 0.3 and 10 μ M (1 μ M for S6, 3 μ M for PRAS40, 3 μ M for GSK3 and 10 μ M for MDM2, respectively) (Figure 3.1C). Phospho S6 inhibition at lower concentrations may reflect the dual AKT and P70S6K pharmacology of AZD5363 (Davies et al., 2012). At high concentrations, such as 100 μ M AZD5363 (at least 10-times the determined IC₅₀) AZD5363 will inhibit other kinases like ROCK1 and ROCK2 as indicated in Table 2.10,

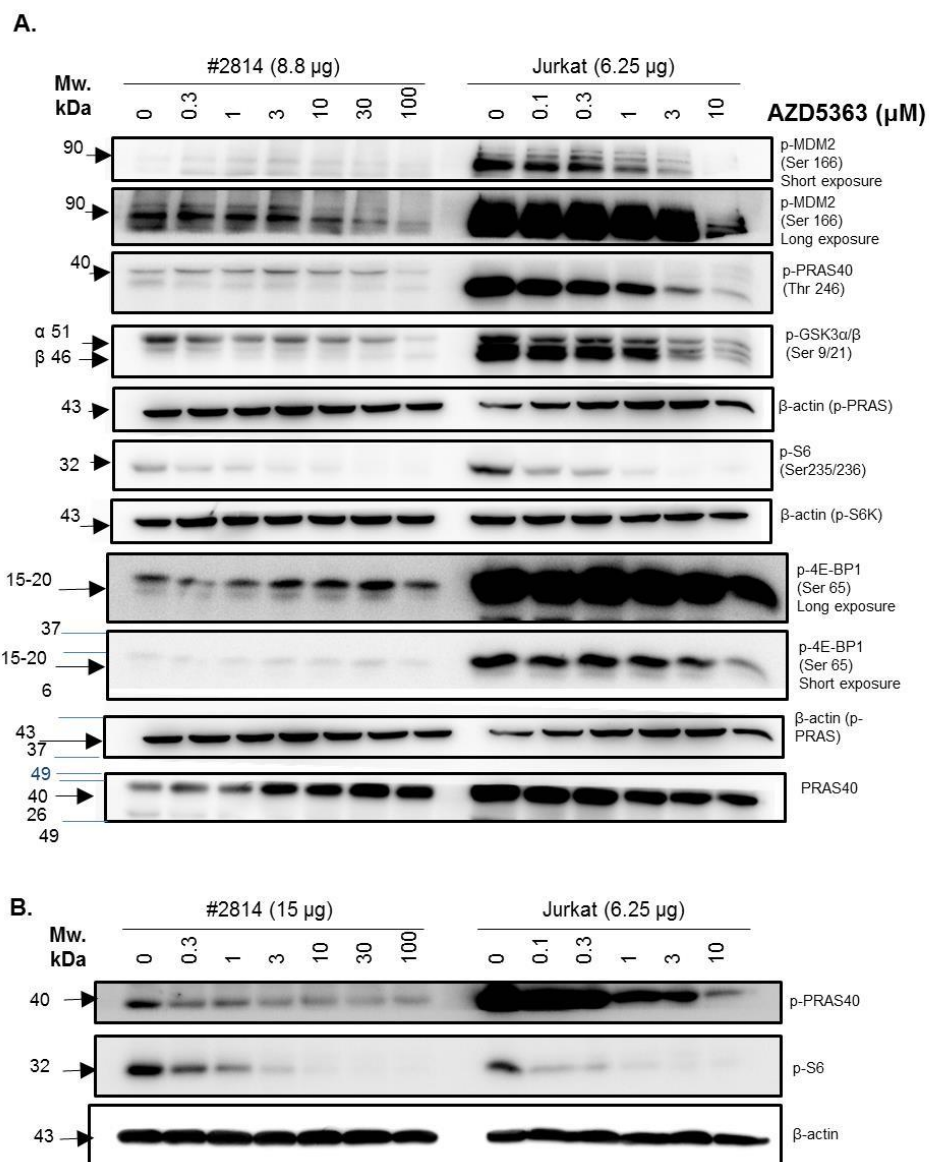
meaning the effect of AZD5363 at these concentrations can not be attributed to the inhibition of AKT alone (Figure 3.1 and Figure 3.2).

Next, cell lysates prepared from two other CLL samples (#2096 and #2103) that had been treated with AZD5363 were also examined for p-GSK3 α/β and total-GSK3 α/β . Again, treatment of CLL cells with AZD5363 resulted in a concentration-dependent reduction of p-GSK3 α/β (Figure 3.1D &E), suggesting that AZD5363 is active in inhibiting AKT. It is of interest to note that the expression of total GSK3 α isoform (51 kDa) was more abundant than that of total GSK3 β isoform (46 kDa) in CLL cells, whereas in Jurkat cells both isoforms were expressed at similar levels (Figure 3.1D &E). Whether this has any biological significance remains to be seen.

As CLL is well known to be a heterogeneous disease, I included more CLL samples with both good (e.g. samples with mutated *IGHV* gene including samples #2521, #1958, #3033 and #2729) as well as poor (e.g. samples containing chromosomal deletion in 11q including sample #2263) prognostic features in the study in order to determine whether AZD5363 is active in all of these CLL cells. Primary CLL cells from these patients were incubated for 24h with AZD5363 at a range of concentrations and then examined for levels of phosphorylated GSK3 α/β and total GSK 3 α/β by Western blotting analysis. As shown in Figure 3.2A-F, AZD5363 consistently decreased the amount of phosphorylated GSK3 α/β in a concentration-dependent manner in all of these CLL samples. Densitometric analysis of signals corresponding to p-GSK3 relative to total GSK3 from all CLL samples examined showed on average an IC₅₀ of about 3 μ M (Figure 3.3A). Taken together, the above results showed that AZD5363 is active and potently inhibits AKT in CLL cells.

It has been reported that AZD5363 can induce phosphorylation of AKT itself in human breast and prostate cancer cells (Davies et al., 2012), acting as a competitive catalytic inhibitor by binding to the ATP binding site of AKT resulting in a hyperphosphorylated but catalytically inactive form of the enzyme (Okuzumi et al., 2009). In order to discover if this was also the case in CLL cells, Phospho-AKT (Ser 473) was also probed for in CLL cells treated with AZD5363. Indeed, AKT was found to be hyperphosphorylated upon the addition of AZD5363 to CLL cells

(Figure 3.2G-I), which was consistent with the findings previously reported (Davies et al., 2012).



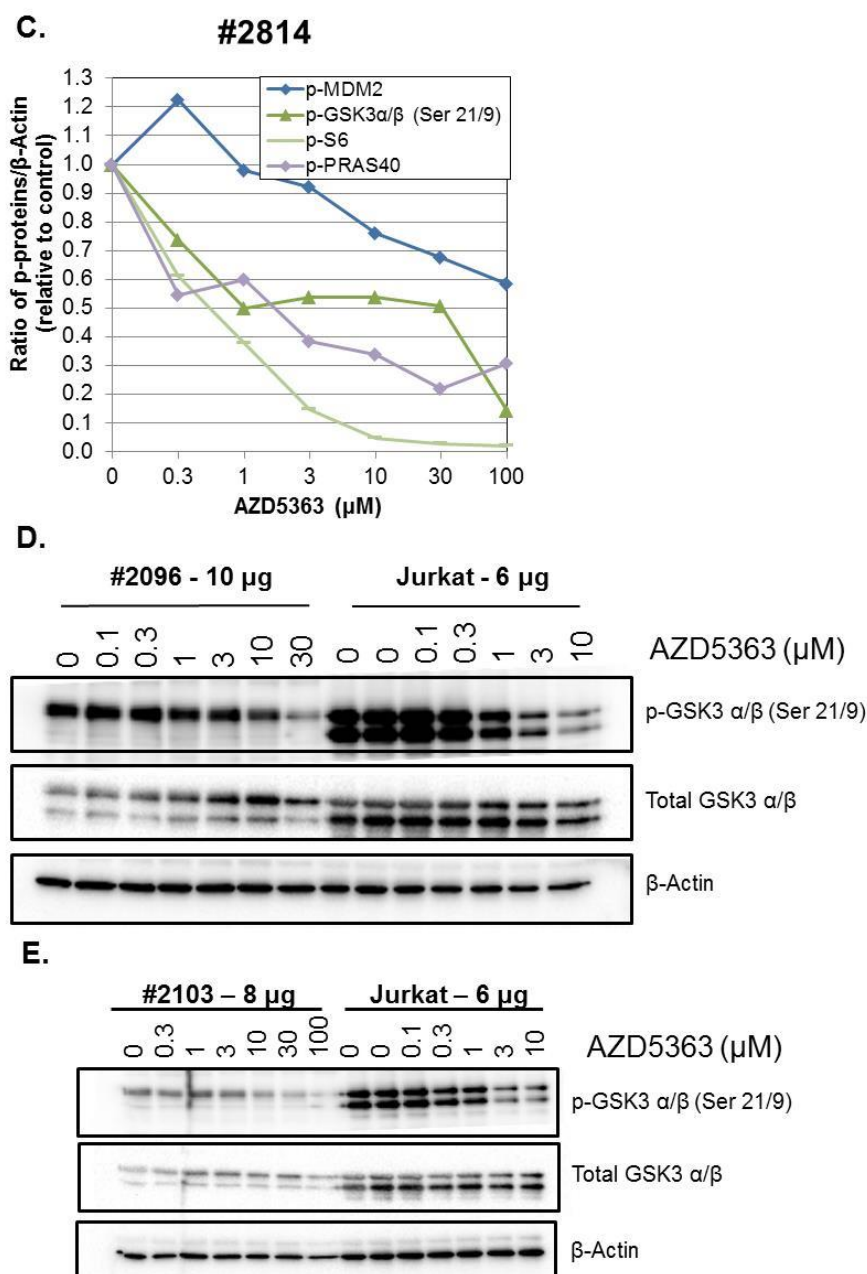


Figure 3.1: Comparison of the biological activity of AZD5363 in primary CLL cells to Jurkat cells

A/B. Effect of AZD5363 in primary CLL cells (sample #2814) versus Jurkat cells.

Jurkat cells (at 1×10^6 cells/ml) and CLL cells (# 2814 at 3×10^6 cells/ml) were incubated for 24h with AZD5363 at the indicated concentrations. Cells were then lysed using RIPA buffer, cellular proteins separated on SDS-PAGE and then examined by western blotting for levels of phosphorylated MDM2 (Ser 166), PRAS40 (Thr246), GSK3α/β (Ser 9/21), S6 (Ser 235/236), 4E-BP1 and AKT (Thr 308). β-actin was used as a loading control.

C. Densitometry analysis.

Densitometry analysis of the signals corresponding to phosphorylated proteins relative to β-actin shown in (A/B) relative to CLL #2814 0 μM AZD5363.

D/E. Effect of AZD5363 in primary CLL cells (samples #2096 and #2103).

Primary CLL cells (#2096 and #2103, at 3×10^6 cells/ml) and Jurkat cells (at 1×10^6 cells/ml) were incubated for 24h with AZD5363 at the indicated concentrations and then examined for levels of phosphorylated GSK3α/β (Ser 9/21), and total GSK3α/β. β-actin was used as a loading control.

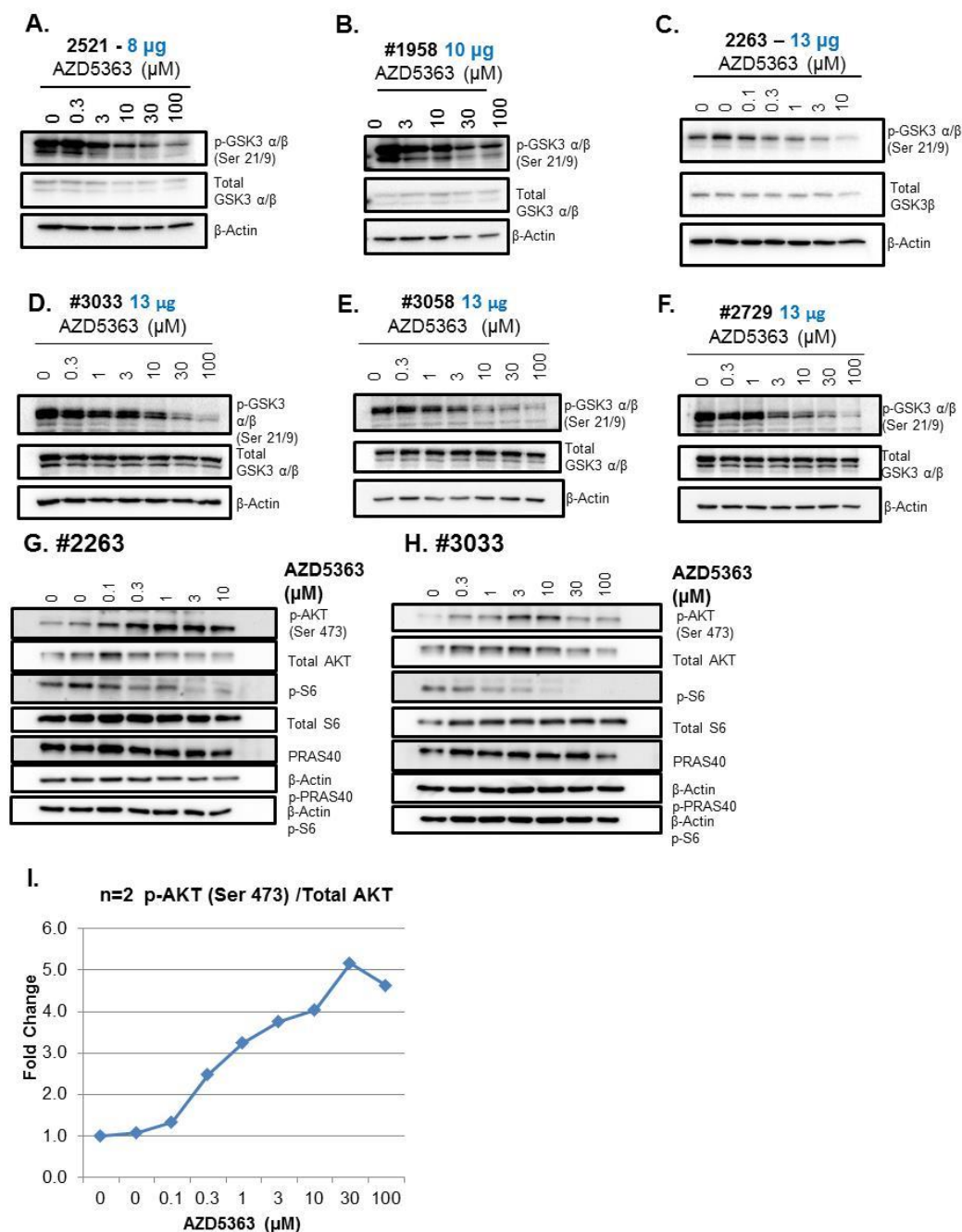


Figure 3.2: Concentration-dependent inhibition of AKT activity by AZD5363 in primary CLL cells

A-F. Primary CLL cells from 6 different patients were incubated for 24h with AZD5363 at the indicated concentrations and then examined for levels of phosphorylated GSK3 α/β and either total GSK 3 β or total GSK 3 α/β . β -actin was used as a loading control. Amount of protein loaded was indicated.

G. Primary CLL cells #2263 were incubated for 24 h with AZD5363 at the indicated concentrations and then examined by Western blotting for levels of phosphorylated AKT (Ser473), total AKT, phospho- and total- S6, phospho- and total-PRAS40. β -actin was used as a loading control.

H. Primary CLL cells #3033 were incubated for 24 h with AZD5363 at the indicated concentrations and then examined by Western blotting for levels of phosphorylated AKT (Ser473), total AKT, phospho- and total- S6, phospho- and total-PRAS40. β -actin was used as a loading control.

I. Densitometry for n=2 (CLL #2263, #3033). Phosphorylated AKT (Ser473)/Total AKT was calculated.

3.2.2 AZD5363 has no effect on the survival of un-stimulated CLL cells

Having established that AZD5363 was active in CLL cells and that the concentration required to achieve 50% inhibition of AKT activity as measured by loss of phosphorylated GSK3 α/β was about 3 μ M (Figure 3.3A), I next examined the effect of AZD5363 on the viability of CLL cells as measured by propidium iodide (PI) exclusion and flow cytometry. As shown in Figure 3.3B, AZD5363 at concentrations up to 30 μ M had no effect on cell viability after 24 h culture under standard conditions. This suggested that AZD5363 was not cytotoxic at the concentrations that inhibited AKT activity. To exclude the possibility that apoptotic cells at early phase of cell death were not detected by the PI method, AZD5363-treated CLL cells were also examined by Western blotting for cleavage of poly(ADP-ribose) polymerase (PARP), a hallmark of apoptosis. However, no increase in PARP cleavage was observed in cells treated with the drug at concentrations up to 30 μ M (Figure 3.3C). Together, these results indicate that AZD5363 has no effect on the survival of unstimulated CLL cells at concentrations that produced pronounced inhibition of AKT activity.

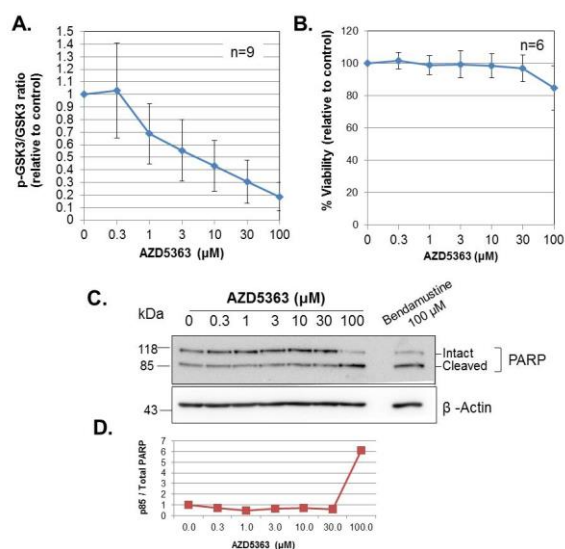


Figure 3.3: AZD5363 at the concentrations that inhibit AKT does not induce cell death

A. Densitometry analysis of the signals corresponding to phosphorylated GSK3 α/β and total GSK3 α/β in CLL cells from nine different patients was performed to quantify the effect of AZD5363 on AKT activity. In this and subsequent graphs, each data point represents the mean \pm SD.

B. Following incubation with AZD5363 at the indicated concentrations for 24 h as in A, viability of CLL cells was measured by propidium iodide (PI) exclusion and flow cytometry, as described in Methods.

C. Following incubation of CLL cells with AZD5363 at the indicated concentrations for 24 h as in A&D, cleavage of PARP was examined by Western blotting. PARP cleavage induced by bendamustine (100 μ M) in CLL cells was used as a positive control (Schwanen et al., 2002).

D. Densitometry of p85 / total PARP over different AZD5363 concentrations, based on figure (C).

3.2.3 AZD5363 does not sensitize unstimulated CLL cells to killing by bendamustine

I next sought to establish whether AZD5363 can sensitize CLL cells to killing by a clinically relevant chemotherapeutic agent. I chose bendamustine owing to its expanding role in the treatment of previously untreated and recurrent CLL (Knauf et al., 2009, Fischer et al., 2011, Fischer et al., 2012).

In agreement with a previous report (Schwanen et al., 2002), incubation of CLL cells with bendamustine over 72 h resulted in a time- and concentration-dependent induction of cell death (Figure 3.4A), with an estimated concentration of the drug that was required to kill 50% of cells (often referred to as LC₅₀) being 100, 20 and 10 μ M at 24, 48 and 72 h, respectively (Figure 3.4A). However, the addition of AZD5363 did not increase the amount of cell death observed (Figure 3.4B).

I was unable to use the Chou and Talalay method to calculate combination indices which indicate whether a drug is additive (CI = 1), synergistic (CI < 1), or antagonistic (CI > 1), because I did not have enough data points, but also I didn't get greater than 50% kill in some of the samples (Chou, 2010, Chou and Talalay, 1984).

To see if AZD5363 increased bendamustine-induced apoptosis, I again used Western blotting to detect PARP cleavage. As expected, treatment with bendamustine resulted in cleavage of PARP in a concentration-dependent manner (Figure 3.4C, lanes 1-7). However, there was little or no increase in cleaved PARP in cells treated with bendamustine plus AZD5363 (Figure 3.4C, lanes 8-14). Taken together, the above results showed that AZD5363 does not sensitize unstimulated CLL cells to bendamustine-induced killing.

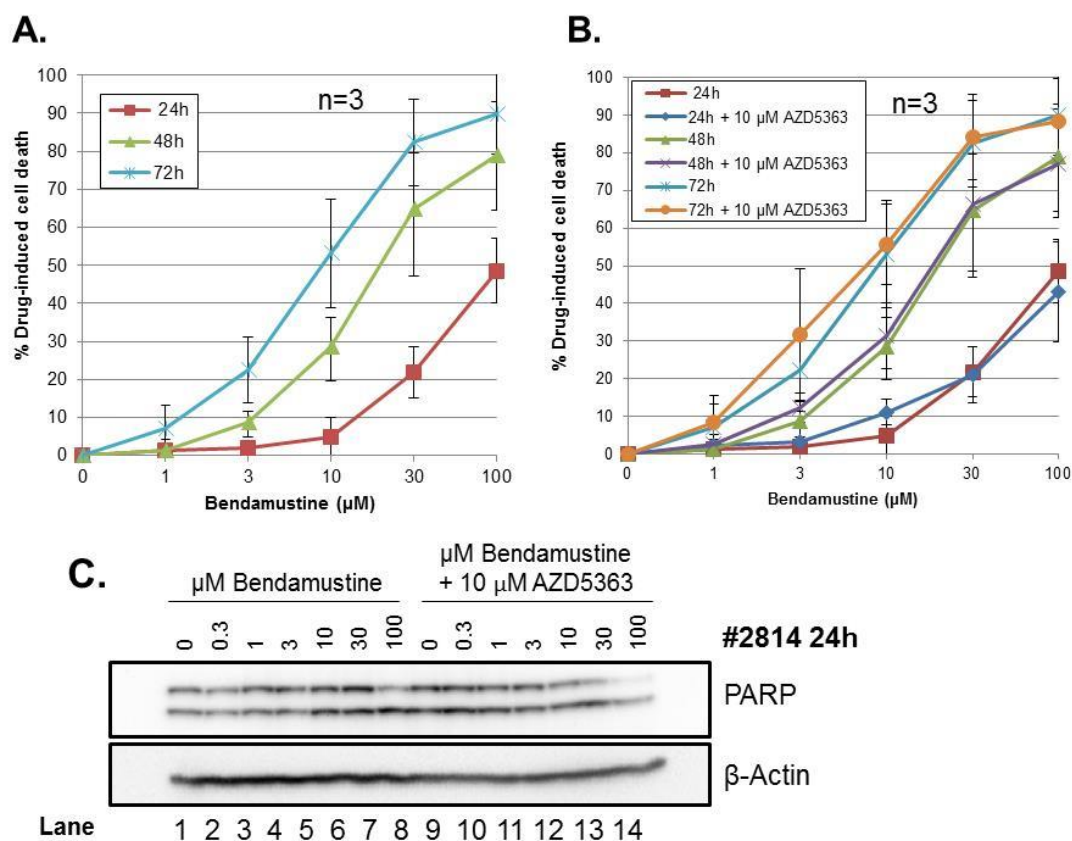


Figure 3.4: Effect of AZD5363 on bendamustine-induced killing under standard culture conditions.

A. Bendamustine-induced killing.

Primary CLL cells were incubated for 72 h with bendamustine at the indicated concentrations in the presence or absence of 10 μM AZD5363. Cell death was examined at 24, 48 and 72 h using the PI/flow cytometry method. Results obtained in the absence of AZD5363. Each data point represents the mean \pm SD.

B. Effect of AZD5363 on bendamustine-induced killing.

Primary CLL cells were incubated for 72 h with bendamustine at the indicated concentrations in the presence or absence of 10 μM AZD5363. Cell death was examined at 24, 48 and 72 h using the PI/flow cytometry method. Each data point represents the mean \pm SD.

C. Effect of bendamustine plus AZD5363 on PARP cleavage

Primary CLL cells were incubated for 24 h with bendamustine at the indicated concentrations in the presence or absence of 10 μM AZD5363 and then analysed for PARP cleavage by Western blotting as in Figure 4C.

3.3 AKT is activated upon CD40 stimulation

Previously, it was reported that the addition of anti-CD40 monoclonal antibodies to CLL cells co-cultured with mouse fibroblasts resulted in a three-fold increase in the level of phosphorylated AKT (at S473) in CLL cells (Cuni et al., 2004). More recently, it was reported that the level of phosphorylated AKT (at S473) was increased by about 1.5-fold in CD19⁺ CLL cells from patients (n = 3) that had been stimulated with 1µg/mL soluble CD40L for 2 hours, when compared with unstimulated cells (Herman et al., 2010). To mimic the T cell-rich lymph node microenvironment, I utilised a co-culture system in which CLL cells are cultured on a monolayer of transfected mouse fibroblasts expressing human CD154. Previous work in the laboratory has shown that co-culture of CLL cells with CD154-expressing fibroblasts not only protected CLL cells from spontaneous cell death but also prevented their killing by therapeutic agents that induce apoptosis through activating either mitochondrial or death receptor-mediated pathways (Zhuang et al., 2014).

I therefore sought to determine if AKT was activated in the CD40-stimulated CLL cells. CLL cells from three different cases were co-cultured with CD154-expressing fibroblasts for 24 h, 48 h or 72 h and then harvested for analysis of AKT activation as indicated by levels of p-AKT (S473 or T308) by Western blotting. As shown in Figure 3.5A and B, compared with cells co-cultured with control fibroblasts, CLL cells co-cultured with CD154-expressing fibroblasts for 24 h expressed increased levels of phosphorylated AKT, by 2.07-fold with phosphorylation at serine 473 (p= 0.04) and by 1.75-fold with phosphorylation at threonine 308. The levels of p-AKT in CLL cells cultured on parental control or CD154-expressing fibroblasts at 48 h and 72 h were also quantified by densitometry analysis. The level of p-AKT at serine 473 was still increased in CLL cells co-cultured with CD154-expressing fibroblasts at these time points, although this increase was no longer statistically significant (Figure 3.5A). The initial increase in the level of phosphorylated AKT at threonine 308 in CD40-stimulated CLL cells at 24 h appeared to disappear at 48 and 72 h time points (Figure 3.5B). In fact, the results from densitometry analysis indicated a decrease in p-AKT (T308) in CD40-stimulated cells at 72 hours (Figure 3.5B).

When comparing the CD40 stimulation back to cells cultured under standard conditions (with no monolayer), we see that the levels of AKT phosphorylation at serine 473 and threonine 308 are comparable. So we can say that at the 24 h time point AKT activation was maintained by CD40 stimulation in CLL cells.

Total AKT appears to be down regulated upon CD154 stimulation at 24 h, 48 h and 72 h time points (Figure 3.5C). Indeed, it has been shown that AKT phosphorylation at serine 473 by mTORC2 promotes a lysine-48-linked polyubiquitination of AKT, resulting in its rapid proteasomal degradation (Wu et al., 2011). In all cases, and definitely in sample #3365, the signals of AKT band seen on the blots in samples prepared from CD40 stimulated CLL cells had moved up to a position of higher molecular weight (Figure 3.5C). This indicated that AKT was phosphorylated upon CD154 stimulation. Consequently, the decrease in total AKT upon CD154 stimulation led to a further increase in the ratio of p-AKT(S473) to total AKT seen in samples prepared from CD40 stimulated CLL cells (Figure 3.5D).

Taken together, the above results showed that AKT activation was maintained in CLL cells following CD40 stimulation.

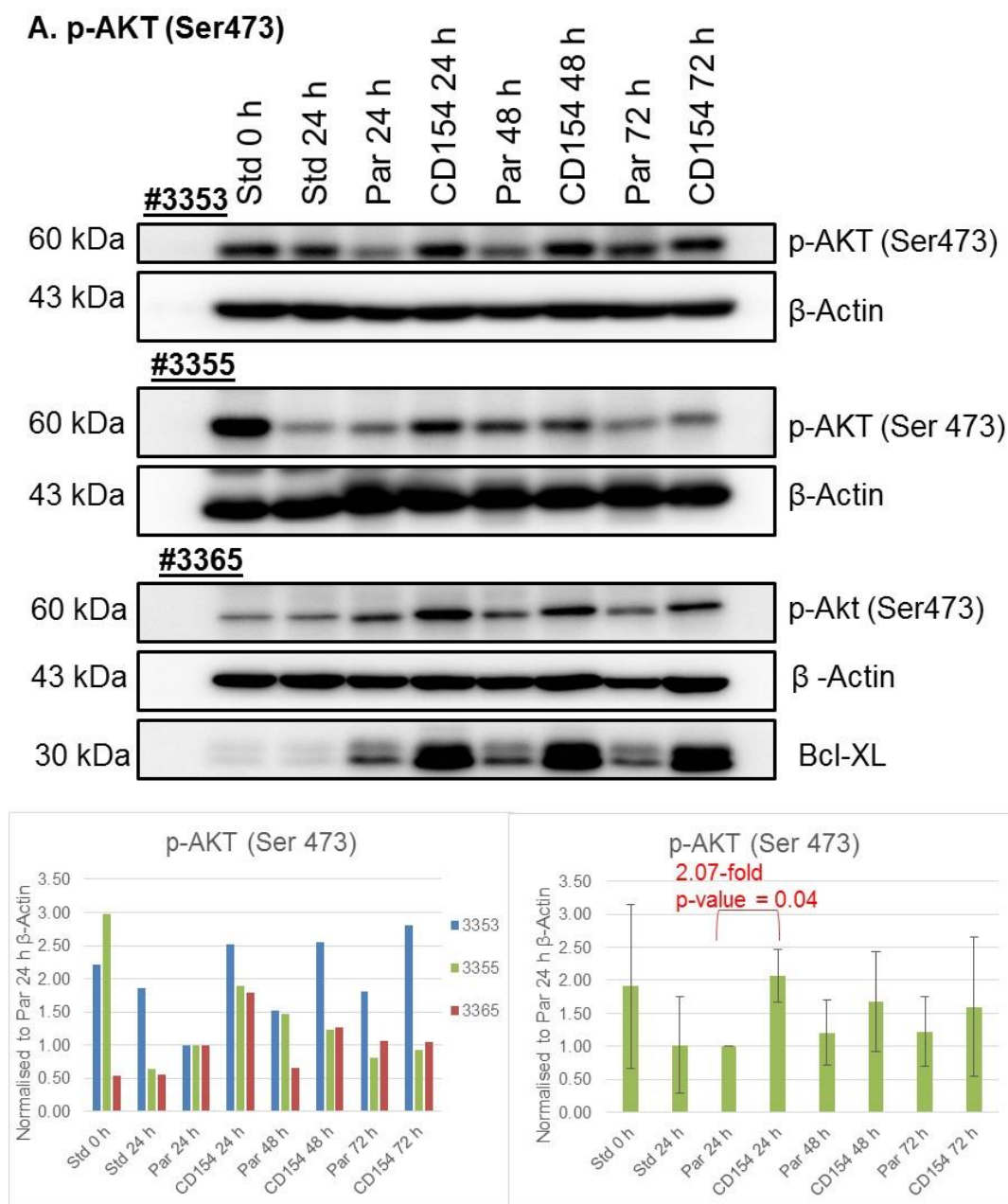
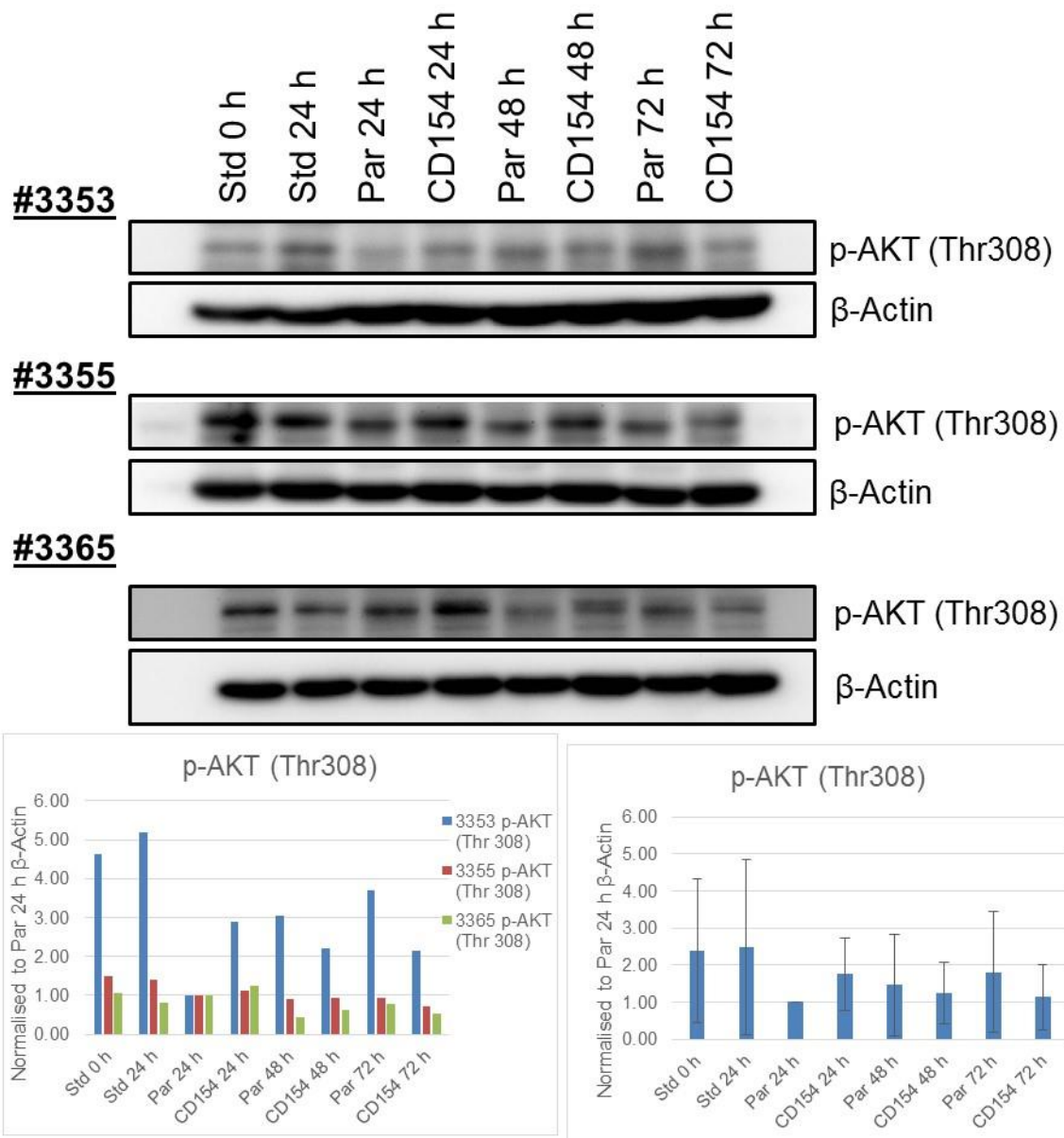


Figure 3.5: AKT activation is maintained in CLL-cells stimulated with CD154.

CLL cells from three cases were thawed and recovered for 1 hour (Standard culture 0 h, Std 0 h), and cultured for 24 hours either alone (Standard culture 24 h, Std 24 h), or co-cultured on parental fibroblasts (Par) or co-cultured with CD154-expressing fibroblasts (CD154) for 24 hours (24h), 48 hours (48h) or 72 hours (72h). Cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in modified RIPA with sonication, protein determination performed and 10 µg of cell lysate loaded onto 10% SDS-PAGE gel, proteins were transferred onto PVDF membrane. The membrane was incubated in primary antibodies overnight, followed by relevant HRP-conjugated secondary antibodies and the western was developed using ECL on a CCD camera. Densitometry was performed using an AIDA image analyser. β-Actin served as a loading control. Bcl-XL served as a positive control for CD154 stimulation.

- A. Western blot of p-AKT (Ser 473) in CLL cases #3353, #3355, #3365 and densitometry of p-AKT (Ser 473) n= 3, normalised to parental 24 hours / β-Actin.**
Graph shows the mean ± SD. Student paired two-tailed t-test were calculated and $p \leq 0.05$ is displayed.

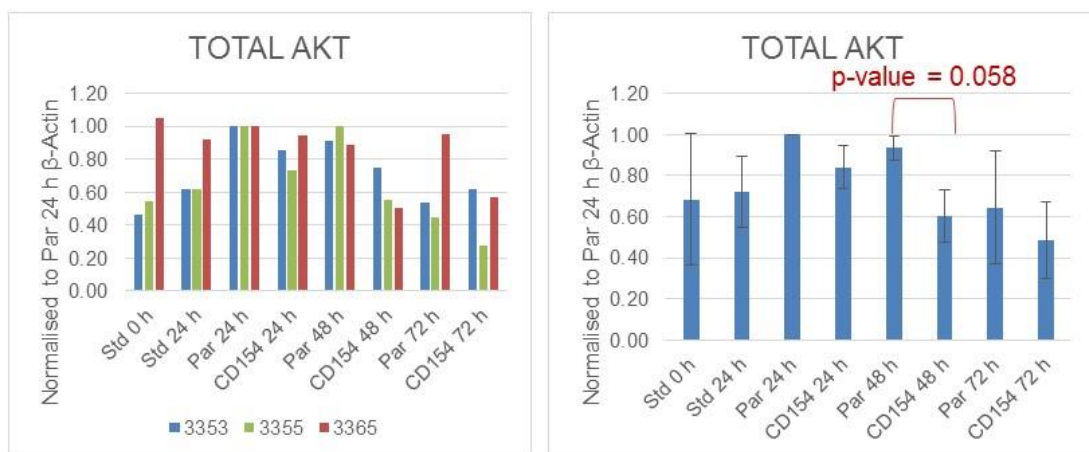
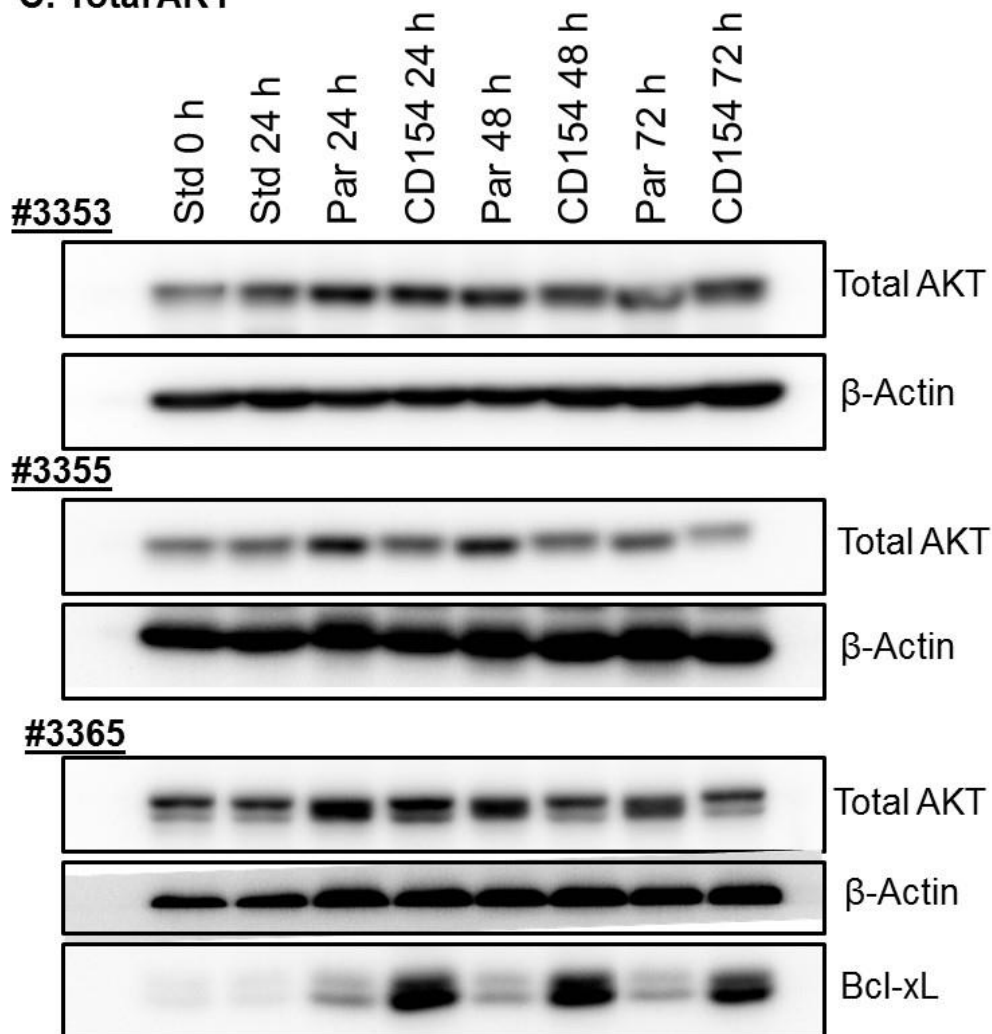
B. p-AKT (Thr308)



B. Western blot of p-AKT (Thr 308) in CLL cases #3353, #3355, #3365 and densitometry of p-AKT (Thr 308) n= 3, normalised to parental 24 hours / β -Actin.

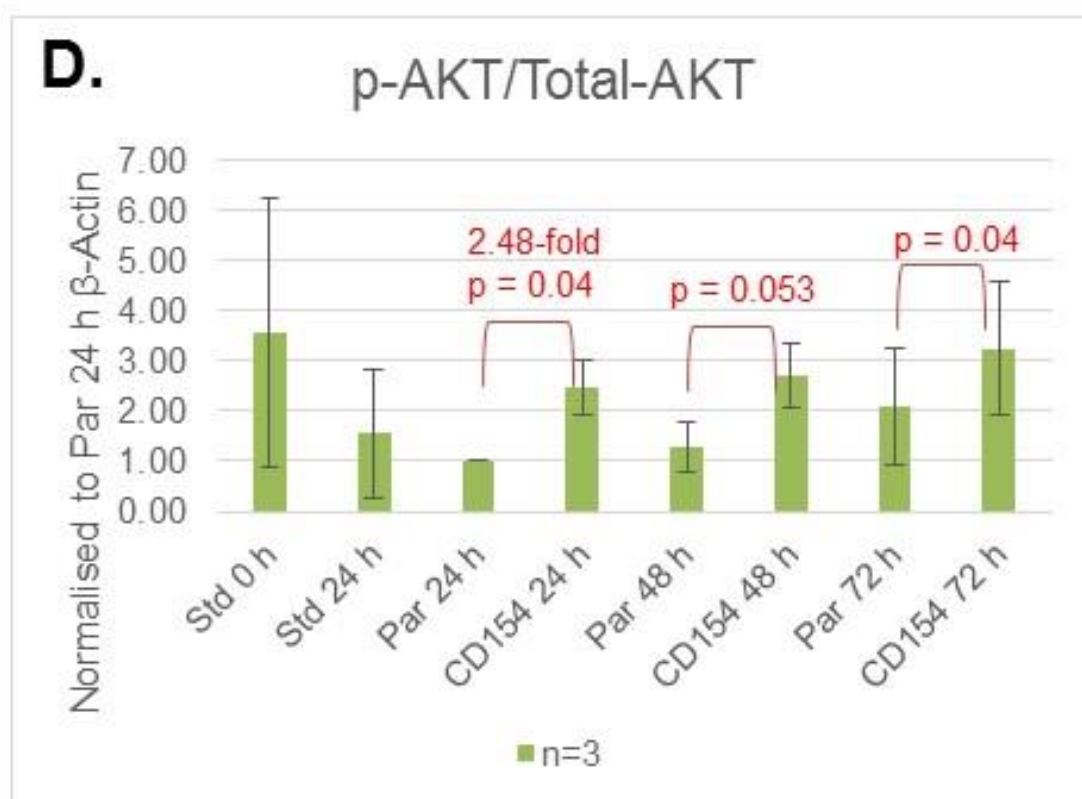
Graph shows the mean \pm SD. Student paired two-tailed t-test were calculated and $p \leq 0.05$ is displayed.

C. Total AKT



C. Western blot of TOTAL AKT in CLL cases #3353, #3355, #3365 and densitometry of TOTAL AKT n= 3, normalised to parental 24 hours / β-Actin.

Graph shows the mean \pm SD. Student paired two-tailed t-test were calculated and $p \leq 0.06$ are displayed.



D. Densitometry of p-AKT/Total-AKT n= 3.

Graph shows the mean \pm SD. Student paired two-tailed t-test were calculated and the p-values \leq 0.06 are displayed.

3.4 Effect of AZD5363 on CD40-stimulated CLL cells

I next determined (a) whether AZD5363 is still active in CLL cells co-cultured with CD154-expressing fibroblasts, and (b) whether, if active, AZD5363 can sensitise the CD40-stimulated CLL cells to drug-induced killing.

3.4.1 CD40-stimulation reduced spontaneous and bendamustine-induced death of CLL cells

CLL cells were cultured under standard conditions, or co-cultured with control or CD154-expressing fibroblasts in the presence or absence of bendamustine at a range of indicated concentrations for 48 hours. As shown in Figure 3.6A, under standard culture conditions a significant proportion of CLL cells underwent spontaneous cell death over 48 hours (an average of 66.5%, green bar) in the absence of bendamustine. Addition of bendamustine resulted in a concentration-dependent increase in cell death, a result consistent with previous observation (Figure 3.4A). In contrast, CLL cells co-cultured with control fibroblasts (red bars) had a significantly

reduced level of spontaneous cell death (an average of 23.0%). Furthermore, CLL cells co-cultured with CD154-expressing fibroblasts (blue bars) had only 14.3% of spontaneous cell death. The difference in spontaneous cell death between CLL cells co-cultured with CD154-expressing fibroblasts and CLL cells cultured under standard conditions was statistically significant ($p=0.03$) (Figure 3.6A).

Regarding drug specific induction of cell death, the addition of bendamustine to CLL cells co-cultured with control fibroblasts (red bars) caused a concentration-dependent increase in cell death, although at a significantly reduced level when compared with that in CLL cells cultured under standard conditions (green bars). At 10 μM concentration, bendamustine induced an average of 5.16% cell death in CLL cells co-cultured with control fibroblasts whereas it induced 28.5% cell death in cells cultured under standard conditions ($p=0.03$) (Figure 3.6B). Similarly, 30 μM bendamustine induced 16.5% cell death in CLL cells co-cultured with control fibroblasts as compared with 64.7% in CLL cells under standard culture conditions ($p=0.02$) (Figure 3.6B).

Nevertheless, a more pronounced protective effect against bendamustine-induced killing was observed in CLL cells that were co-cultured with CD154-expressing fibroblasts. Such co-cultured CLL cells (blue bars) were completely protected from cell death induced by bendamustine at 10 and 30 μM concentrations (Figure 3.6B). There was only 6% cell death detected in CLL cells treated with 100 μM bendamustine (Figure 3.6B). This was in stark contrast to 65.5% cell death induced by the drug at the same concentration in CLL cells co-cultured with control fibroblasts ($p=0.01$) (Figure 3.6B). These results therefore clearly demonstrated that CD40 stimulation effectively protected the CLL cells from spontaneous and drug-induced cell death.

The effect of bendamustine on either the control or CD40L-expressing fibroblasts viability was not assessed (nor on their irradiated forms), however the fibroblasts morphology was not significantly altered by addition of bendamustine as observed under light microscope.

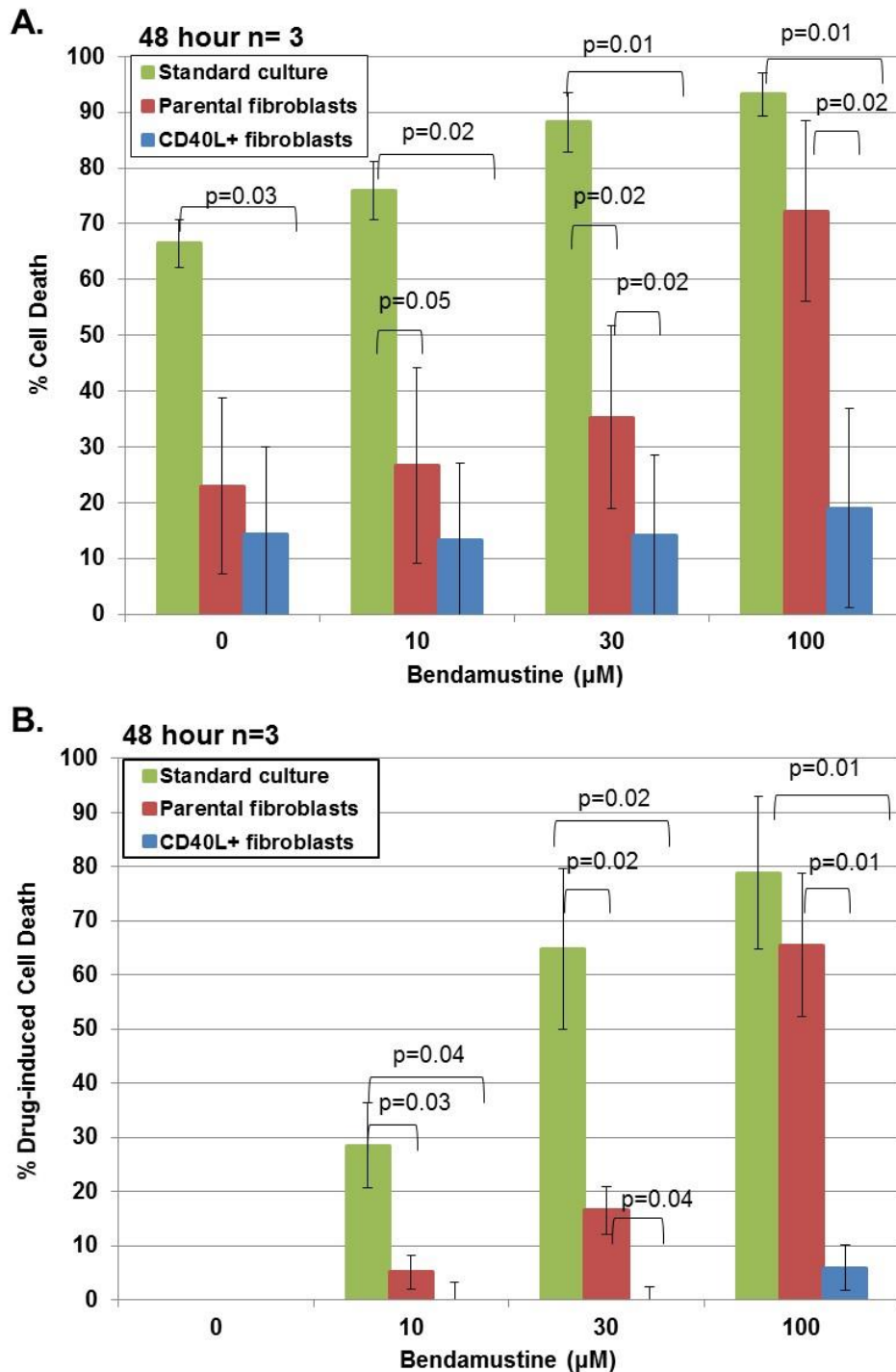


Figure 3.6: CD40 stimulation inhibits spontaneous and bendamustine-induced CLL cell death.

- CLL cells were cultured in standard conditions or co-cultured with control or CD40L-expressing (CD40L+) fibroblasts for 48 h in the presence or absence of bendamustine at the indicated concentrations and then examined by PI staining and flow cytometry for cell death. Three CLL cases were examined, the mean of $n=3 \pm$ standard deviation are displayed.
- The percentage of drug-induced cell death was calculated as: $100 \times [(\% \text{ death of drug-treated cells} - \% \text{ death of untreated cells}) / (100 - \% \text{ death of untreated cells})]$.

p-values were calculated using the student two-tailed paired t-test; p-values ≤ 0.05 are displayed. Graph shows the mean \pm SD.

3.4.2 AZD5363 is still active in CLL cells co-cultured with CD154-expressing cells

Having shown that AKT was activated in CD40-stimulated CLL cells ([section 3.3](#)), I went on to determine whether AZD5363 inhibited AKT activity in these cells. As shown in Figure 3.7A-D, levels of p-GSK3 α/β were readily detectable in CLL cells co-cultured with parental fibroblasts or CD154-expressing fibroblasts. In keeping with the results obtained in un-stimulated CLL cells, treatment with AZD5363 led to a concentration-dependent inhibition of AKT, as measured by de-phosphorylation of GSK3 α/β , with 10 μ M AZD5363 achieving greater than 50% inhibition (Figure 3.7D).

At the same time, I have also examined the phosphorylation status of other downstream substrates of AKT including MDM2, PRAS40 and S6 in CLL cells on co-cultures with or without AZD5363. As shown in [Appendix section 2.2](#), Appendix Figure 3, AZD5363 induced a concentration-dependent de-phosphorylation of these substrates with 10 μ M AZD5363 achieving greater than 50% reduction in phosphorylation of most of these AKT substrates. Based on the above results, I therefore concluded that AZD5363 was still able to inhibit AKT in CLL cells co-cultured with CD154-expressing fibroblasts, with 10 μ M AZD5363 achieving greater than 50% inhibition.

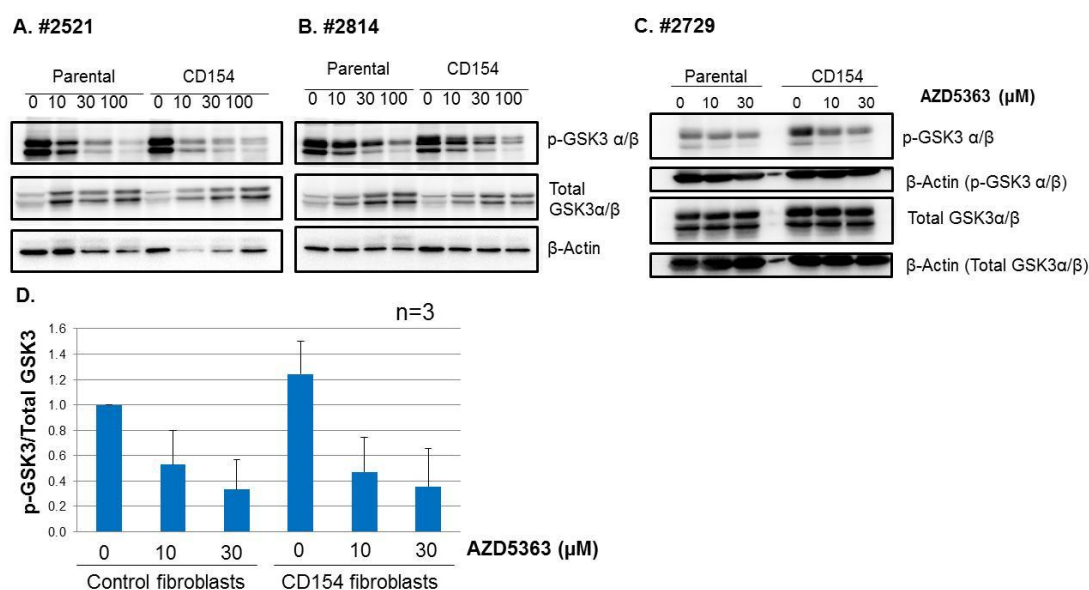


Figure 3.7: Effect of AZD5363 on AKT activity in CD40-stimulated CLL cells

CLL cells were co-cultured for 24 h with control or CD154-expressing (CD154+) fibroblasts in the presence or absence of AZD5363 at the indicated concentrations and then examined by Western blotting. **A-C.** Western blotting for levels of phosphorylated GSK3α/β as a measure of AKT activity. Total GSK3α/β and β-Actin were used as loading controls.

D. Densitometric analysis of the signals corresponding to phosphorylated GSK3α/β and total GSK3α/β in CLL cells (n=3). Graph displays the mean ± SD (as in Figure 7A-C).

3.4.3 AZD5363 reduces the viability of CD40-stimulated CLL cells and sensitizes them to bendamustine-induced killing

Next, I examined the effect of AZD5363 on the baseline viability and bendamustine-induced killing of CLL cells co-cultured on monolayers of control or CD154-expressing fibroblasts for 24 and 48 h.

CLL cells appear to have less baseline death at 48 hours compared to 24 hours on control fibroblasts (6.24% compared to 20.60%) and CD40L-expressing fibroblasts (3.25% compared to 11.0%), the exact reasoning for this is unknown. Though one might guess that cells which have been thawed out, which are slowly apoptosing are still present at 24 hours, whilst by 48 hours they have lysed and are no longer detectable, leaving only the surviving CLL cells. Perhaps more information could have been gleaned had an Annexin V- PI assay been used.

Consistent with the results seen previously (Figure 3.6), the viability of untreated CLL cells co-cultured with parental fibroblasts for 24 and 48 h remained high (Figure 3.8A and B). At 48 h, more than 90% of cells were viable (Figure 3.8B) a much higher proportion than was observed under standard culture conditions (Figure

3.6). These cells were also noticeably more resistant to bendamustine-induced killing. Only about 40% cell death was observed at 48 h in these co-cultured CLL cells when treated with 100 μ M bendamustine (Figure 3.8B). This was in stark contrast with 80% cell death detected in similarly treated CLL cells cultured under standard conditions (Figure 3.6).

More importantly, baseline cell viability and resistance to bendamustine-induced killing were further increased in CLL cells co-cultured with CD154-expressing fibroblasts, as compared with CLL cells co-cultured with control fibroblasts at 24 h and 48 h time points (Figure 3.8A and B). At 48h, there was less than 20% cell death observed in CLL cells co-cultured with CD154-expressing fibroblasts in the presence of 100 μ M bendamustine (Figure 3.8B), whereas over 40% cell death was observed in CLL cells co-cultured with control fibroblasts (Figure 3.8B) and about 80% cell death detected in CLL cells cultured under standard conditions when treated with the same concentration of bendamustine (Figure 3.6).

Next, I wanted to find out if AZD5363 can sensitise co-cultured CLL cells to killing by bendamustine. Treatment with 10 μ M AZD5363 alone resulted in a small but significant increase in baseline death of CLL cells co-cultured with either control or CD154-expressing fibroblasts over a 48 h time period (Figure 3.8A and B). Whilst the increase was not significant at 24h, by 48h 10 μ M AZD5363 increased cell death from 6.24% to 12.62% ($p=0.034$) in CLL cells co-cultured with control fibroblasts and from 3.25% to 7.58% ($p=0.026$) in CLL cells co-cultured with CD154-expressing fibroblasts.

10 μ M AZD5363 increased bendamustine-induced cell death. At 48 h, 10 μ M AZD5363 increased cell death from about 14.5% to 26.2% ($p=0.005$) in CLL cells co-cultured with control fibroblasts and from about 4.3% to 13.91% ($p=0.010$) in CLL cells co-cultured with CD154-expressing fibroblasts when treated with 30 μ M bendamustine (Figure 3.8B). Also at 48 h, 10 μ M AZD5363 increased cell death induced by 100 μ M bendamustine from 42.0% to 64.8% ($p=0.002$) in CLL cells co-cultured with control fibroblasts and from about 18.5% to 49.7% ($p=0.010$) in CLL cells co-cultured with CD154-expressing fibroblasts (Figure 3.8B).

Taken together, the findings that AZD5363 increased both baseline and bendamustine-induced cell death, suggest that AZD5363 blocks pro-survival signals generated from control and CD154-expressing fibroblasts, implicating AKT as one of the mediators of stromal cell-derived pro-survival signals.

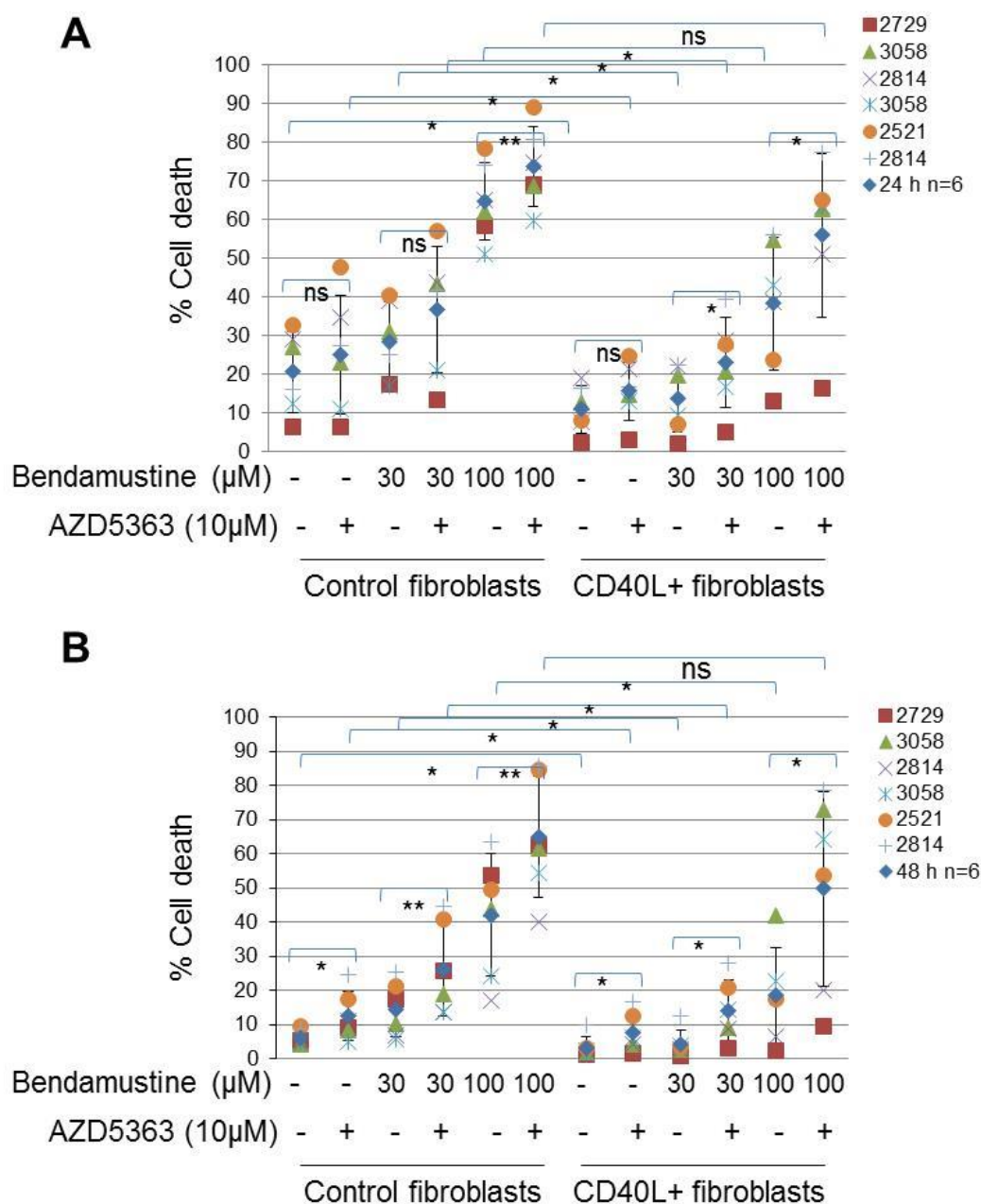


Figure 3.8: Effect of AZD5363 on the survival and bendamustine-induced killing of CD40-stimulated CLL cells

CLL cells (n=6: #2729, #2521, #3058 x 2 and #2814 x 2) were co-cultured with control or CD154-transfected fibroblasts in the presence or absence of bendamustine at the indicated concentrations with or without AZD5363 (10 μM) for 24 h (A) and 48 h (B), and cell death was examined using PI staining. n=6 is the mean \pm SD. A two-tailed paired student t-Test was performed to determine the statistical significance of the difference between the two groups of data.

* refers to $P < 0.05$, ** refers to $P < 0.01$, and ns refers to not significant.

3.5 Role of p53 in sensitising CD40-stimulated CLL cells to bendamustine following AKT inhibition

AKT is known to phosphorylate MDM2 and phosphorylated MDM2 is known to be important for its p53-inhibitory activity (Mayo and Donner, 2001, Gottlieb et al., 2002). Treatment of CLL cells with the AKT inhibitor A-443654 has been shown to decrease levels of p-MDM2 and induce p53 activation (Zhuang et al., 2010). By reducing levels of p-MDM2 (Figure 3.1A), AZD5363 could have a similar effect on p53 levels and augment the p53 activation that underpins the cytotoxic effect of bendamustine (Leoni et al., 2008, Fischer et al., 2011) and other types of chemotherapy (Gonzalez et al., 2011).

I thus sought to address the question of whether the additional killing effect of AZD5363 to chemotherapy is p53-dependent. To do this, I used CLL samples from patients with known defects in p53, e.g. those with chromosomal deletion in 17p, and performed the same experiment to test if AZD5363 will still enhance bendamustine-induced killing in these cells.

3.5.1 Effect of bendamustine plus AZD5363 on viability of CD40-stimulated CLL cells containing 17p deletion

CLL cells with 17p deletion were cultured alone or co-cultured with control or CD154-transfected fibroblasts in the presence or absence of bendamustine at the indicated concentrations with or without AZD5363 (10 μ M) for 24 h (Figure 3.9A and Figure 3.10A) and 48 h (Figure 3.9B and Figure 3.10B), and cell death was examined using PI staining and flow cytometry.

As expected, the percentage of bendamustine-induced cell death in standard culture conditions (green bars, Figure 3.9A&B) is significantly reduced in CLL cells containing 17p deletion, compared to that in cells with no known defects in p53 function (red bars, Figure 3.9A&B). In the cells with 17p deletion, treatment with 30 μ M and 100 μ M of bendamustine for 48 hours induced 6.6% and 23.3% of cell death, respectively (green bars, Figure 3.9B), whereas 64.7% and 78.8% of cell death were observed in cells with no known defects in p53 function (red bars, Figure 3.9B). This clearly shows the induction of cell death by bendamustine is largely p53 dependent. Sample #2103 has 95% 17p deleted cells whereas sample #3325 has 77%

17p deleted cells, whilst the 17p deletion clone size for sample #3361 is unknown. Case #3325 was more responsive to the bendamustine-induced cell death than case #2103 at 24 hours (purple compared to blue bars, Figure 3.9A), this may in part be explained by the lower clone size with 17p deletions in #3325 (77% compared to 95% 17p-). With no bendamustine, case #3325 also seemed more sensitive to AZD5363 at 24 hours than case #2103 (12.1% versus 5.2%, purple compared to blue bars, Figure 3.9A). This suggests that the 17p deletion clone size has an impact on sensitivity to both bendamustine and AZD5363, but a larger sample size would be required for this to be conclusively proven.

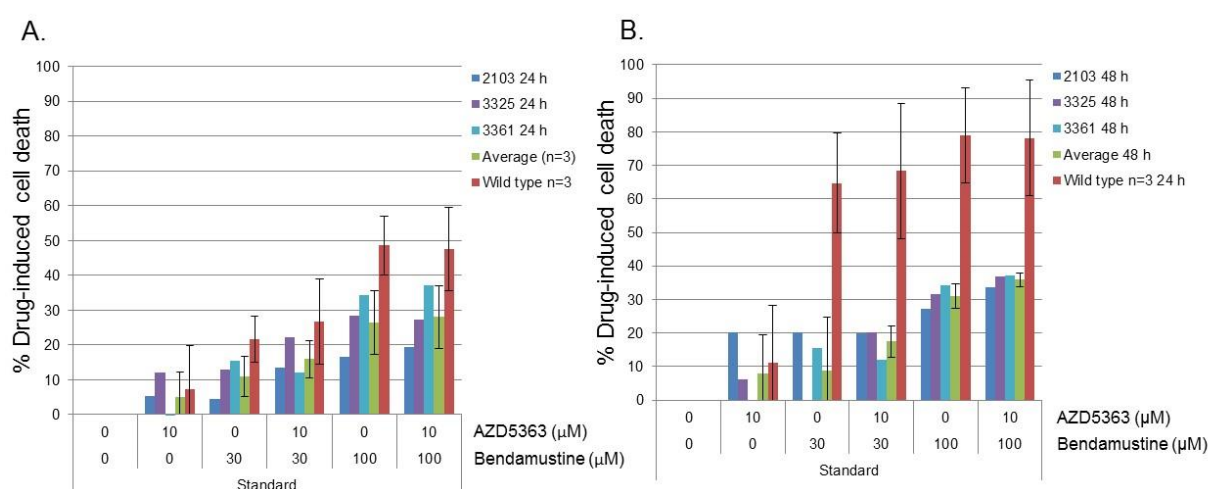


Figure 3.9: Induction of cell death by AZD5363 plus bendamustine in CLL cells with chromosomal deletion in 17p.

CLL cells with chromosomal deletion in 17p (or wild-type) were cultured alone in the presence or absence of bendamustine at the indicated concentrations with or without AZD5363 (10 μ M) for 24 h (A) and 48 h (B), and cell death was examined using PI staining. The percentage of drug-induced cell death at 24 h (A) and 48 h (B) was calculated as: $100 \times [(\% \text{ death of drug-treated cells} - \% \text{ death of untreated cells}) / (100 - \% \text{ death of untreated cells})]$. $n=3$ is the mean \pm SD. A two-tailed paired student t-test was performed to determine the statistical significance of the difference between the two groups of data. Any p-values less than $P < 0.05$ are displayed.

Next I examined the effect of bendamustine-induced cell death in CLL cells with 17p deletion that were co-cultured with control fibroblasts (green bars, Figure 3.10A&B), and compared it to that in CLL cells with no known defects in p53 function (red bars, Figure 3.10A&B). I observed that the amount of bendamustine-induced cell death at 24 h (both at 30 and 100 μ M concentrations) is significantly less in cells with 17p deletion (14.7% and 24.0% respectively) as compared to that in cells with no known defects in p53 function (28.3% and 64.8% respectively). At 48 h, 11.5% and 10.9% cell death were observed in CD40-stimulated CLL cells with 17p deletion that were treated with 30 and 100 μ M bendamustine respectively, as compared to 14.54% and

42.03% cell death respectively observed in cells with no known defects in p53 function. This again clearly shows the cell death induced by bendamustine is mainly p53-dependent.

Finally, I examined the effect of bendamustine in CD40-stimulated CLL cells with 17p deletion (green bars, Figure 3.10A&B) and compared it to that in CD40-stimulated cells with no known defects in p53 function (red bars, Figure 3.10A&B). Again, the amount of bendamustine-induced cell death was significantly less in cells with 17p deletion than those without the defect. At 24 h time point, 30 μ M bendamustine induced 9.8% cell death in cells with 17p deletion versus 13.8% in cells without the defect, and 100 μ M bendamustine induced 11.9% cell death versus 38.2%, respectively. At 48 h, 100 μ M bendamustine induced 7.1% cell death versus 18.5%, respectively. This clearly shows that the cell death induced by bendamustine is p53-dependent.

However, the sensitising effect of AZD5363 to killing by bendamustine was also observed in CD40-stimulated CLL cells with chromosomal deletion in 17p. Upon parental fibroblasts at 48 h with 100 μ M bendamustine, the addition of 10 μ M AZD5363 increased cell death from 10.9% to 22.6%. Upon CD154 fibroblasts at 48 h with 100 μ M bendamustine, the addition of 10 μ M AZD5363 increased cell death from 7.1% to 21.9% (green bars, Figure 3.10A&B). Given that 10 μ M AZD5363 increases cell death so much at 100 μ M bendamustine in the co-culture setting, one might conclude that it is not working in a p53-dependent manner, since these cells have a chromosomal deletion in 17p (as measured by FISH), we assume that they do not express functional p53. Though the p53 responses were not evaluated in these cells prior to evaluation with bendamustine, and this would ideally be performed in the future.

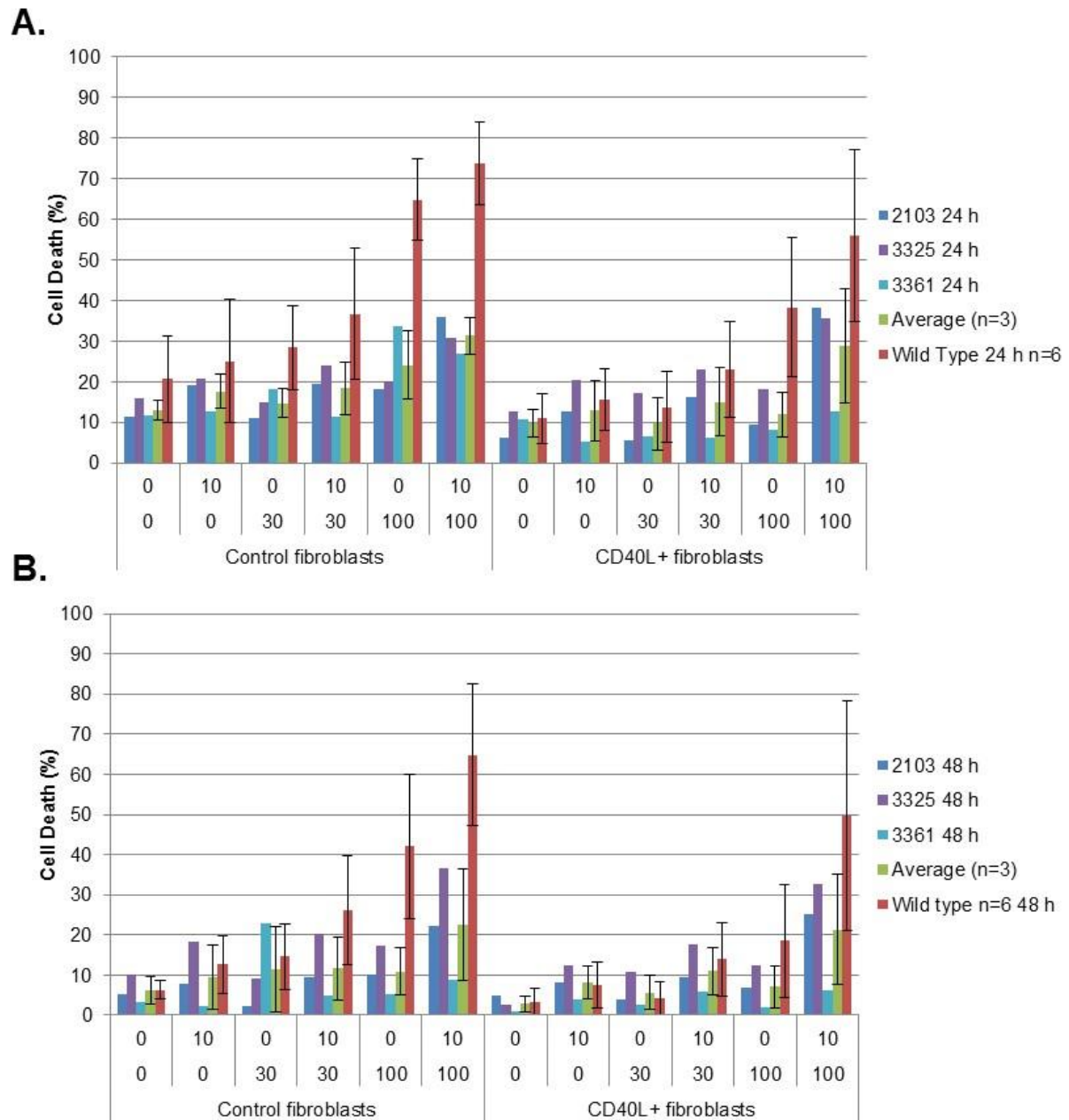


Figure 3.10: Induction of cell death by AZD5363 plus bendamustine in CD40 stimulated CLL cells with chromosomal deletion in 17p.

CLL cells with chromosomal deletion in 17p (or wild-type) were co-cultured with control or CD40L+-transfected fibroblasts in the presence or absence of bendamustine at the indicated concentrations with or without AZD5363 (10 μ M) for 24 h (A) and 48 h (B), and cell death was examined using PI staining. Average (n=3) shows the mean \pm SD. Wild type n=6 shows the mean \pm SD. A two-tailed paired student t-test was performed to determine the statistical significance of the difference between the two groups of data. Any p-values less than $P < 0.05$ are displayed.

3.6 Summary

The AKT inhibitor AZD5363 is active and potently inhibits AKT in CLL cells, inhibiting the phosphorylation of several well characterised downstream substrates of AKT, on average by 50% at 3 μ M. However, AZD5363 appeared not to be cytotoxic at the concentrations that inhibit AKT since AZD5363 at concentrations up to 30 μ M had no effect on cell viability after 24 h culture under standard conditions. AZD5363 did not sensitize un-stimulated CLL cells to bendamustine-induced killing.

AKT activation was maintained by CD40 stimulation in CLL cells.

The AKT inhibitor, AZD5363 inhibited AKT in CD40-stimulated CLL cells. I showed that AZD5363 reduced the viability of CLL cells co-cultured with CD154-expressing fibroblasts and sensitized these CD40-stimulated CLL cells to bendamustine-induced killing.

I wondered whether the extra killing following addition of AZD5363 to bendamustine in CD40-stimulated CLL cells was caused by the AKT inhibition-induced upregulation of p53 due to loss of MDM2. To answer this question, I examined the killing effect of bendamustine following AKT inhibition (AZD5363) of CD40-stimulated CLL cells with chromosomal deletion in 17p. Addition of 10 μ M AZD5363 increased cell death induced by 100 μ M bendamustine in CD40-stimulated CLL cells with chromosomal deletion in 17p, even though these cells are not sensitive to bendamustine (100 μ M) alone. This suggests that additional killing by AZD5363 to bendamustine does not involve p53 since these cells do not express wild type p53.

3.7 Discussion

The AKT inhibitor AZD5363 at concentrations up to 30 μ M effectively inhibited AKT activity, as measured by loss of phosphorylation of several well characterised substrates of AKT, but had no effect on viability of CLL cells after 24 h culture under standard conditions. These results are in contrast to observations reported in CLL cells treated with another AKT inhibitor, MK-2206. MK-2206 at 1 μ M concentration was shown to reduce AKT phosphorylation (Ding et al., 2013). However, 8 μ M MK-2206 was required to induce 50% apoptosis after 72 h in CLL cells (n= 37), with almost no cell death induced in normal PBMC cells (n= 5) (Ding

et al., 2013). These investigations also showed that the killing effect of MK-2206 was independent of common prognostic indicators (Ding et al., 2013).

There are many factors that may contribute to these contrasting findings. Firstly, it is worth noting that AZD5363 and MK-2206 have differing modes of action (Figure 3.11). AZD5363 binds the kinase domain, whereas MK-2206 binds the PH domain. In light of this difference, the methods used to measure the activity of these drugs are different. In the case of MK-2206, its activity was measured by examining phosphorylation of AKT at serine 473, whereas for AZD5363, its activity was measured by examining the phosphorylation status of its downstream substrates, such as GSK3, since AZD5363 binds to the ATP binding site of AKT resulting in a hyperphosphorylated but catalytically inactive form of the enzyme (Okuzumi et al., 2009).

Secondly, the concentration of MK-2206 needed to induce 50% apoptosis in CLL cells over 72 h (8 μ M) is eight-fold higher than the concentration required to inhibit phosphorylation of AKT at serine 473 (1 μ M). The possibility that cell death induced by the higher concentration of MK-2206 was due to off-target effects cannot be excluded. Whilst the relative selectivity of MK-2206 for AKT has been tested against 250 other kinases, MK-2206 may also bind to other PH or PX-domain containing proteins such as BTK or SGK3, especially at high concentrations.

The third and most plausible explanation for the different effect on CLL survival comes from the recent report of a kinase-independent function of AKT that also promotes cancer cell survival. Vivanco and colleagues observed similar differences in toxicity using MK-2206 and the ATP-competitive AKT inhibitors GSK690693 and GDC0068 (Vivanco et al., 2014). The allosteric inhibitor MK-2206 induced significantly more cell death than the ATP-competitive AKT inhibitors in several cell lines with *MET* amplifications, *HER2* amplification and/or *PIK3CA*-mutation, despite less complete inhibition of AKT substrate phosphorylation at these drug concentrations.

Vivanco and colleagues also observed that both ATP-competitive and allosteric AKT inhibitors were able to induce near-complete proliferation arrest, but had differing effects on cell survival (Vivanco et al., 2014). They showed that the greater cell death induced by the allosteric AKT inhibitor was due to specific binding of MK-

2206 to an amino acid within the PH domain of AKT. Mutation of this residue (W80A) abolished MK-2206-induced cell death.

In addition, Vivanco and colleagues revealed a novel kinase-independent function of AKT that also promotes cancer cell survival. They showed that cancer cells expressing catalytically inactive AKT (AKT K179M) were partially protected from MK-2206-induced cell death (Vivanco et al., 2014). Therefore, prevention of recruitment of AKT to the plasma membrane by MK-2206, but not by AZD5363, might result in these differences in toxicity, either alone or in combination with bendamustine (Figure 3.11).

I showed that AKT activation was maintained by CD40 stimulation in CLL cells. Co-culture of CLL cells with CD154-expressing fibroblasts also effectively protected the leukaemic cells from spontaneous and bendamustine-induced cell death (Figure 3.6). CD40 stimulation has been shown to cause resistance to chemotherapeutic agents such as fludarabine and dexamethasone in CLL cells (Romano et al., 1998, Zhuang et al., 2014), but this is the first demonstration of CD40 stimulation of CLL cells resulting in resistance to bendamustine.

The AKT inhibitor, AZD5363, inhibited AKT activity in CD40-stimulated CLL cells as it inhibited phosphorylation of many downstream substrates of AKT. I also showed that 10 μ M AZD5363 reduced the viability of CLL cells co-cultured with control fibroblasts by 6.38% and CD154-expressing fibroblasts by 4.33% after 48 h. This suggests that AKT plays a role in mediating the protective effect of CLL cells by co-culture and CD40 stimulation. However, despite AKT phosphorylation appearing to be increased two-fold by CD40 ligation ([section 3.3](#)), enhancement of spontaneous cell death by AZD5363 was not significantly greater in CLL cells co-cultured with CD154-expressing fibroblasts than in cells co-cultured with parental fibroblasts, suggesting that AKT probably does not play a major role in mediating the pro-survival effects of CD40 ligation. This notion is in keeping with the currently accepted view that NF- κ B plays a dominant role in mediating CD40-activated survival signalling (Schattner, 2000, Furman et al., 2000).

AZD5363 did sensitize CLL cells co-cultured with control or CD154-expressing cells to bendamustine-induced killing. At 48 h, 10 μ M AZD5363 increased cell death in CLL cells from 14.5% to 26.2% when co-cultured with control fibroblasts and from 4.3% to 13.91% in CLL cells co-cultured with CD154-expressing fibroblasts, when treated with 30 μ M bendamustine. At 48 h, 10 μ M AZD5363 increased cell death from 42.0% to 64.8% in CLL cells co-cultured with control fibroblasts and from 18.5% to 49.7% in CLL cells co-cultured with CD154-expressing fibroblasts when treated with 100 μ M bendamustine. Since AZD5363 increased killing by bendamustine in CLL cells co-cultured with both control and CD154-expressing fibroblasts, this indicates that AKT partially mediates pro-survival signalling induced by both control cells and CD40 stimulation. However, AZD5363 (10 μ M) restored killing by bendamustine in CD40-stimulated CLL cells to the level of bendamustine-induced killing in CLL cells co-cultured with control fibroblasts, suggesting that

AKT may be one of the mediators of CD40-induced resistance to bendamustine in CLL cells.

I also investigated whether the additional killing of CD40-stimulated CLL cells to bendamustine following AKT inhibition by AZD5363 was dependent upon p53. The p53 responses were not evaluated in these cells prior to evaluation with bendamustine, as most CLL patients with 17p deletion also carry *TP53* mutations in the remaining allele (>80%) (Zenz et al., 2008b, Rossi et al., 2009, Dicker et al., 2009). CLL cells with deletion of chromosome 17p were less sensitive than wild type cells to bendamustine alone; however, the addition of 10 μ M AZD5363 increased cell death induced by 100 μ M bendamustine in these cells under co-culture conditions. This suggests that the increased sensitivity to bendamustine by AZD5363 in co-cultured CLL cells occurs in a p53-independent manner since these cells were assumed not to contain a functional p53. Ideally, p53 responses should also be assessed in these cases.

Experiments combining 10 μ M AZD5363 and dexamethasone were difficult to interpret, due to the small sample size, and that the CLL samples chosen were largely insensitive to killing by dexamethasone.

For future work, I would be interested in repeating both these experiments with a lower dose of AZD5363, e.g. 3 μ M, which was shown to be the IC₅₀ for inhibiting GSK3 phosphorylation.

If I had more time, I would like to examine changes in expression of p53 or MCL1 upon treatment with AZD5363. I would expect MCL1 to be down-regulated upon treatment with AZD5363, since inhibition of AKT by A-443654 or siRNA resulted in a loss of MCL1 (Zhuang et al., 2010, de Frias et al., 2009).

Although A-443654 was previously shown to induce p53 activation in CLL cells (Zhuang et al., 2010, de Frias et al., 2009), I predict that p53 would not be activated upon AKT inhibition by AZD5363, since the increase in bendamustine-induced killing was also observed in CLL cells with chromosomal deletions in 17p.

Chapter 4 : The role of AKT in antigen-independent proliferation of CLL cells

4.1 Background and objectives.

As described in the Introduction chapter, AKT promotes cell growth, cell cycle progression and proliferation. AKT mediates cell growth and protein synthesis via mTORC1 resulting in increased cell size (see [section 1.3.4.3](#)). AKT promotes cell cycle progression by negatively regulating CDKs in order to allow cell cycle entry in the following ways: firstly by directly phosphorylating the CDKs leading to their degradation and secondly by phosphorylating forkhead family of transcription factors (FOXOs). FOXOs normally transcribe CDKs, however, phosphorylation of FOXOs in the nucleus by AKT causes them to translocate to the cytosol, resulting in a loss of transcriptional activation of CDKs (see [section 1.3.4.1.3](#)).

Having shown in the previous chapter that AKT activation is maintained upon CD40 stimulation, I next investigated the role of AKT in CLL-cell proliferation induced by stimulation with CD40 + IL-4 or IL-21. I also sought to establish whether inhibiting AKT reduces CLL-cell proliferation.

To do this, I used two novel AKT inhibitors with differing modes of action, namely AZD5363 and MK-2206. AZD5363 binds the kinase domain and has high specificity for all three isoforms of AKT (Davies et al., 2012), whereas MK-2206 is an allosteric inhibitor of AKT, which binds the PH-domain of the kinase, preventing it from localising to the plasma membrane. MK-2206 has high specificity for AKT1 and AKT2 (Hirai et al., 2010). I reasoned that the two AKT inhibitors, AZD5363 and MK-2206 may inhibit cell division/proliferation in CLL cells when co-cultured with CD154-expressing fibroblasts in the presence of certain cytokines.

As cell growth (increase in cell size) usually occurs prior to the cell division, I also monitored changes in size of these stimulated CLL cells based on alterations in forward light scatter properties by flow cytometry.

4.2 CD40 + IL-4 induced CLL-cell proliferation.

4.2.1 Induction of proliferation of CLL cells co-cultured with CD154-expressing fibroblasts + IL-4

To induce CLL cells to proliferate, I co-cultured them with CD154-expressing fibroblasts in the presence of IL-4. To measure cell division, CLL cells were pre-incubated with the fluorescent dye CFSE and monitored for reduction of fluorescence intensity from CFSE by flow cytometry. As expected, CLL cells were induced to divide in a time-dependent manner (Figure 4.1). However, as shown in Figure 4.1, the time required for CLL cells to reach a comparable rate of cell division varied from case to case. For example, CLL cells from case #2814 required seven days to accumulate 33.5% divided cells (Figure 4.1A&B), whereas CLL cells from case #3058 needed ten days to record 28.2% divided cells (Figure 4.1C). On average on day 4 or 5, 9.12 % of the CLL cells had divided, by day 6 15.97 % of the CLL cells had divided, and by day 7 22.86 % of the CLL cells had divided (Figure 4.3C).

The purity of the CLL cells used for this experiment was not assessed since the white blood cell counts and lymphocyte counts were high, see table below:

	WBC (x 10 ⁹ /L)	Lymphocyte count (x 10 ⁹ /L)	% Lymphocytes
Normal reference range	4.00-11.0	1.0–3.0	~25
#2814	196.1		Not known
#3058	51.3	48.4	94.34
#2729	37.2	30.6	82.25

Phorbol myristate acetate (PMA) which stimulates protein kinase C (PKC) and which leads to CLL cells proliferate and differentiate into antibody secreting cells could've have been used as a positive control (Ghamlouch et al., 2014). The phenotype of the cells, which showed reduced CFSE staining (those that had proliferated) was not assessed in this experiment. In later experiments using IL-21, the phenotype of the cells, which showed reduced CFSE staining (those that had proliferated) was assessed by either single CD19 positivity or dual staining for CD5 and CD19 to check that the proliferating cells were CLL cells (see [section 4.8](#), Figure 4.17 and Figure 4.18).

4.2.2 AKT inhibition by AZD5363 inhibits proliferation of CLL cells induced by CD154 + IL-4.

Since AKT was activated in CLL cells co-cultured with CD154-expressing fibroblasts ([Section 3.3](#), Figure 3.5), I wondered to what extent AKT contributes CLL proliferation induced by CD40L + IL-4. To this end, I used the AKT inhibitor AZD5363. As shown in Figure 4.2, treatment of co-cultured CLL cells with 10 μ M AZD5363 inhibited the reduction of fluorescence intensity of CFSE in CLL cells from all three samples examined, indicating an anti-proliferative effect of the AKT inhibitor (Figure 4.2).

Quantitative analysis of the CFSE data showed that the presence of 10 μ M AZD5363 significantly reduced the proliferation of CLL cells induced by CD154 + IL-4 on day 6 and day 7 to $39.3 \pm 31.7\%$ and $30.9 \pm 20.5\%$, respectively (Figure 4.3A-D). Importantly, AZD5363 had no effect on cell viability in this culture system (Figure 4.3E), indicating that the anti-proliferative effect of AZD5363 was specific and not merely a consequence of cytotoxicity. Together, the above results suggest that AKT is required for CLL-cell proliferation induced by CD40 + IL-4.

In these experiments, we don't know that the effect is specific to AKT, since AZD5363 does indeed inhibit other kinases such as have 'off-target effects', such as P70S6K, PKA, ROCK1 and ROCK2 (see section [2.2.2.3 AKT inhibitors](#)). However, later on, when stimulating CLL cells to proliferate with CD154 + IL-21, a second AKT inhibitor with a different mode of action, MK-2206 was also used to verify that the effects seen were due to AKT inhibition ([section 4.6](#)).

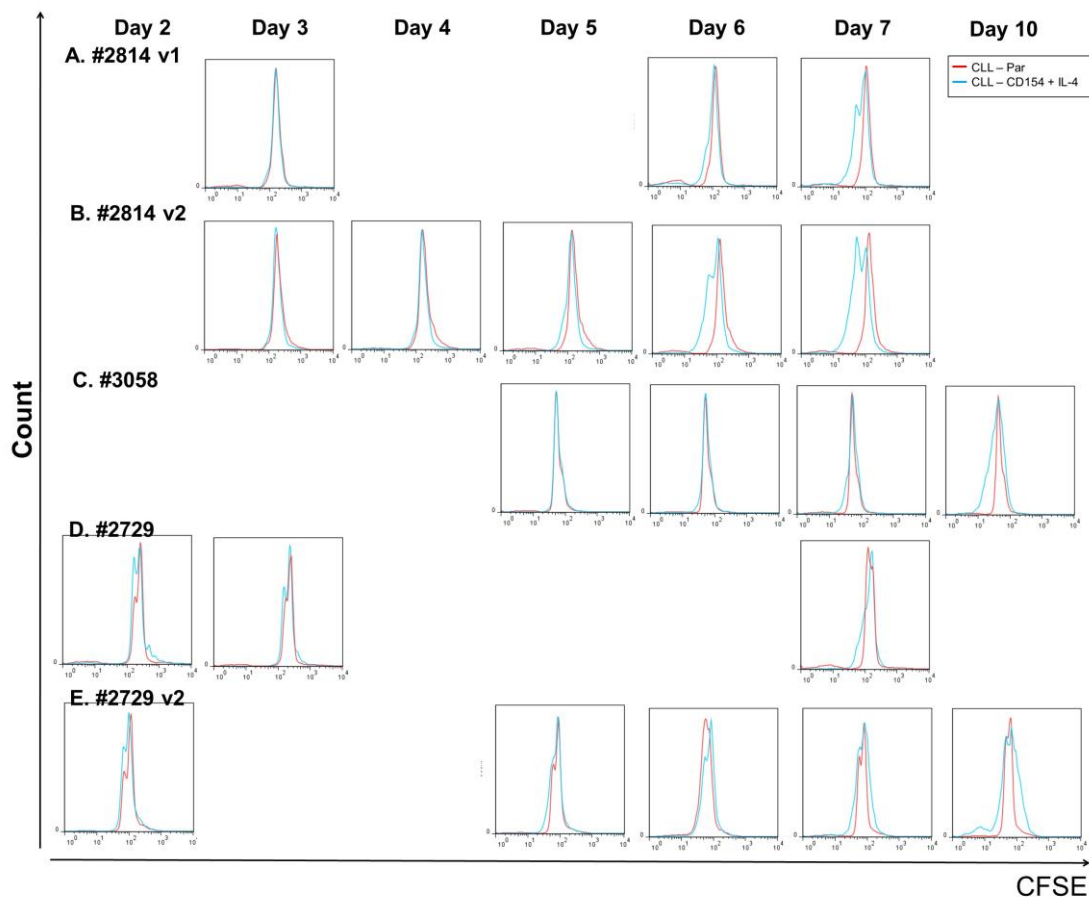


Figure 4.1: CD154 + IL-4 induced CLL-cell proliferation

CFSE-stained primary CLL cells were induced to proliferate when co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-4 (10 ng/ml). Proliferation was detected by flow cytometry as loss of fluorescence intensity as described in the Methods. CLL cells co-cultured with parental fibroblasts were used as a negative control.

- A. #2814 v 1.
- B. #2814 v 2.
- C. #3058.
- D. #2729.
- E. #2729 v 2.

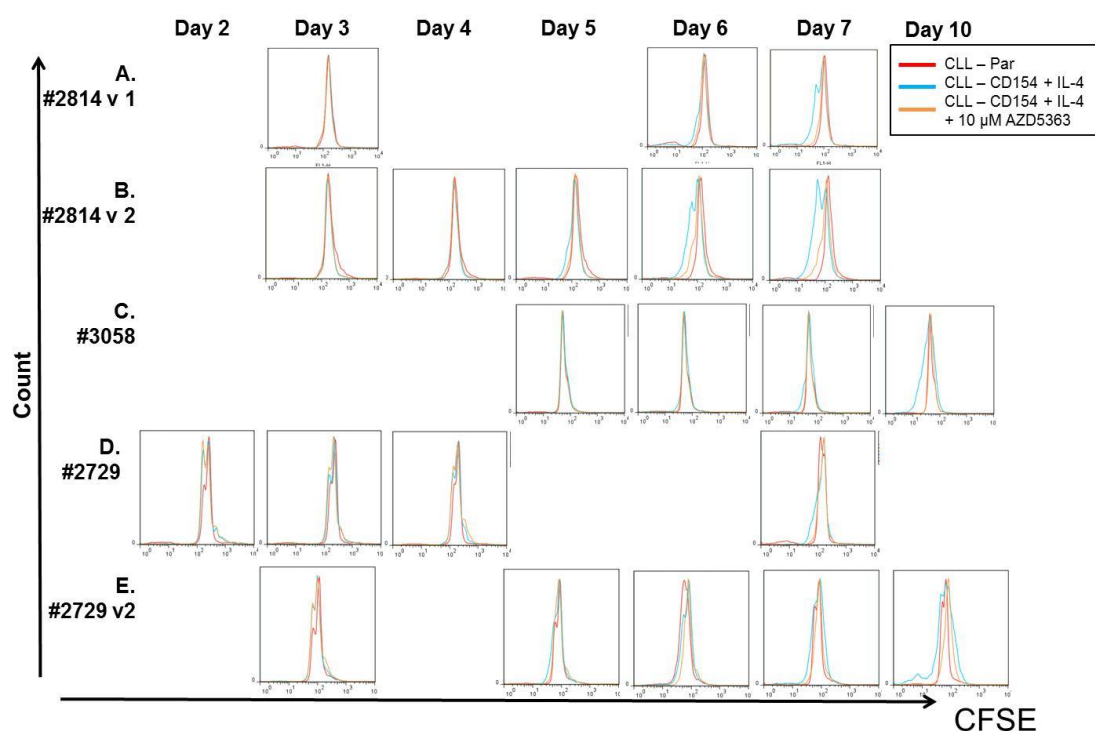


Figure 4.2: CD154 + IL-4-induced CLL-cell proliferation inhibited by AZD5363

CFSE-stained primary CLL cells were induced to proliferate when co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-4 (10 ng/ml). Proliferation was detected by flow cytometry as loss of fluorescence intensity as described in the Methods. CLL cells co-cultured with parental fibroblasts were used as a negative control. Experiments were performed in the presence or absence of 10 μ M AZD5363.

- A. 2814 v 1.
- B. #2814 v 2.
- C. #3058.
- D. #2729.
- E. #2729 v 2.

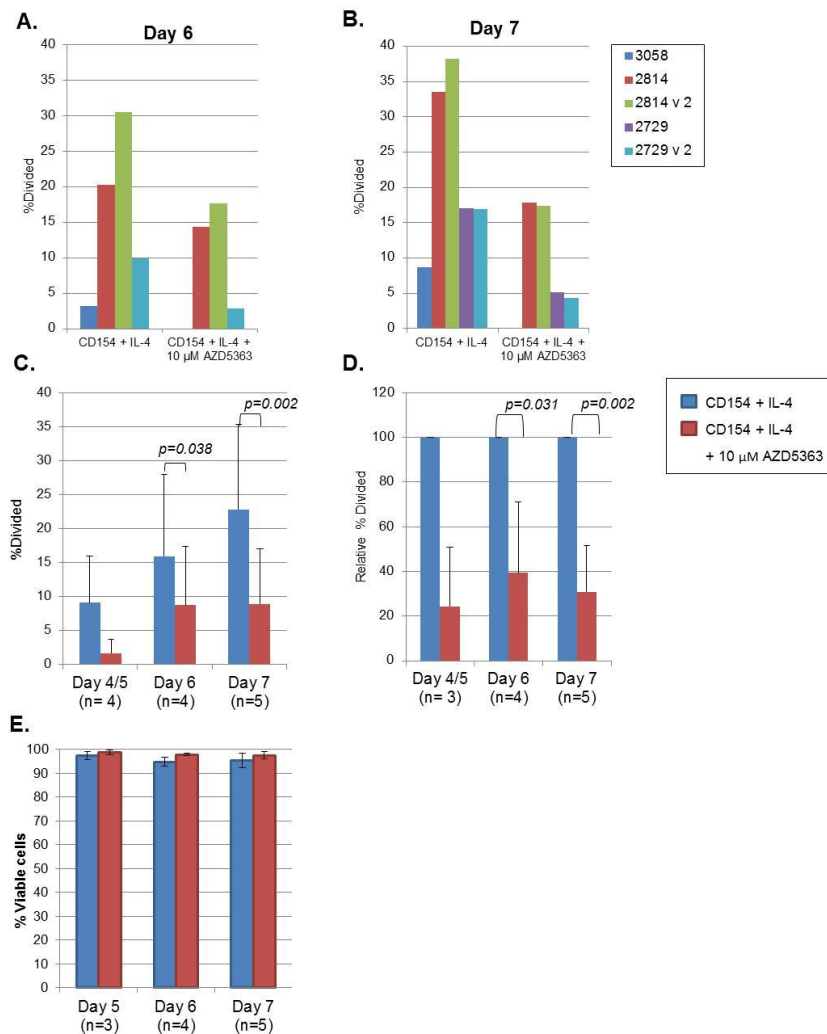


Figure 4.3: Proliferation and cell viability after CD154 + IL-4 induced CLL proliferation and drug treatment.

CFSE-stained primary CLL cells were induced to proliferate when co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-4 (10 ng/ml) for up to seven days. Proliferation was detected by flow cytometry as loss of fluorescence intensity and analysed for the percentage divided using FlowJo. Experiments were performed in the presence or absence of 10 μ M AZD5363.

- The respective values obtained in the absence and presence of 10 μ M AZD5363 for day 6 were 20.3% vs. 14.4% (case #2814), 30.5% vs. 17.7% (case #2814v2), 3.15% vs. 0% (case #3058) and 9.93% vs. 2.81% (case #2729).
- The respective values obtained in the absence and presence of 10 μ M AZD5363 for day 7 were 33.5% vs. 17.8% (case #2814), 38.2% vs. 17.4% (case #2814v2), 8.7% vs. 0% (case #3058) and 17.0% vs. 5.1% (case #2729), and 16.9% vs. 4.37% (case #2729v2).
- % Divided average \pm standard deviation for day 4/5 (n=4), day 6 (n=4) and day 7 (n=5). Student paired two-tailed t-test were calculated.
- In order to see the effect of 10 μ M AZD5363, the number of post-mitotic daughter cells upon CD154 +IL-4 stimulation was normalised to 100%, to create a 'relative % divided'. 10 μ M AZD5363 inhibited proliferation to $39.3 \pm 31.7\%$ to that of the control on day 6 and to $30.9 \pm 20.5\%$ to that of the control on day 7. Student paired two-tailed t-test were calculated
- The viability of the CLL cells on day 7 was measured using the PI/flow cytometry method. Average viability (%) n=3 average (\pm Standard deviation). Student paired two-tailed t-test were calculated.

4.2.3 Effect of AKT inhibition on cell size of CD40-stimulated CLL cells

Whilst monitoring cell division in CFSE-stained CLL cells using flow cytometry, I also observed a time-dependent increase in forward light scatter (FSC) in cells that were co-cultured with CD154-expressing fibroblasts (Figure 4.4). As FSC is typically indicative of the size of a cell (Ormerod, 2000a), the increase in the FSC thus suggests an increase in size of the CD40-stimulated cells. Since AKT has also been reported to regulate cell growth (indicated by increase in cell size) through mTOR and its downstream targets S6K1 and 4EBP1/eIF4E (Edinger and Thompson, 2002, Fingar et al., 2002), I sought to determine if the increase in the size of CLL cells co-cultured with CD154-expressing fibroblasts was also mediated by AKT.

As shown in Figure 4.4A-D, CLL cells co-cultured with CD154-expressing fibroblasts displayed increased cell size (as measured by increased FSC on flow cytometry) in a time-dependent fashion. This increase was statistically significant. In contrast, CLL cells co-cultured with control fibroblasts did not show increase in cell size (Figure 4.4).

(Note: that CLL cells in standard culture alone were not observed, since after three days in standard culture conditions many CLL cells apoptose).

Addition of AZD5363 (10 μ M) significantly inhibited the increase in size of CD40-stimulated cells (Figure 4.5A-D). The inhibition occurred as early as 24 h after incubating CLL cells with AZD5363 (Figure 4.5C-D). As I have shown in the previous chapter that 10 μ M AZD5363 inhibited AKT in CLL cells co-cultured with CD154-expressing fibroblasts ([Section 3.4.2](#), Figure 3.7), the inhibition of increase in cell size of CD40-stimulated cells by AZD5363 seen here was likely as a result of inhibition of AKT activity by AZD5363.

(Note: since the CLL cells plated upon parental cells did not increase in size over time, AZD5363 was not added to them to observe if the size was decreased. There was no control for CLL cells cultured on the parental fibroblast cells with AZD5363. We assumed that AZD5363 did not affect the size of non-proliferating cells).

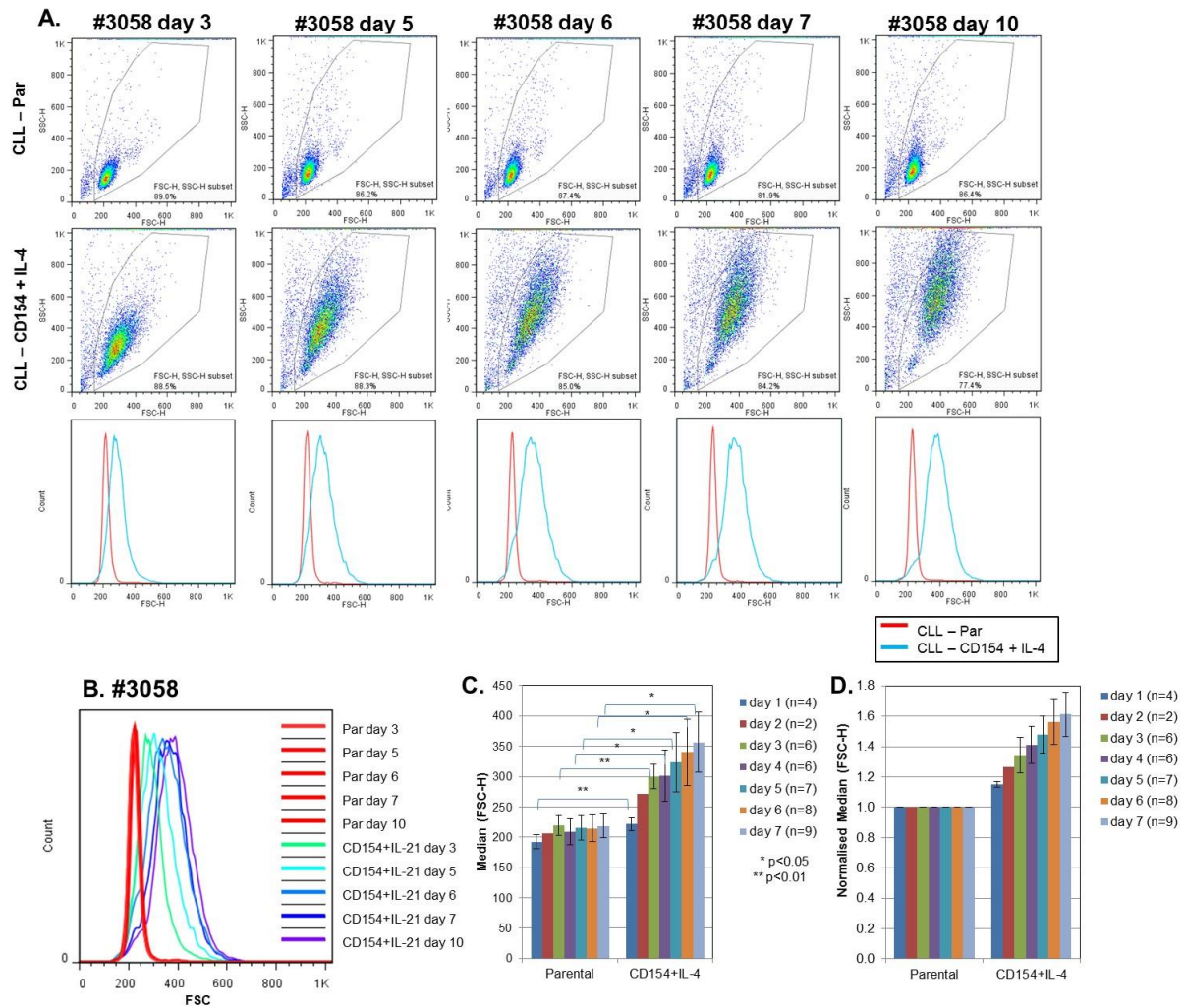


Figure 4.4: Effect of CD40-stimulation on size of CLL cells.

- FSC-SSC plots by flow cytometry show the increase in cell size upon CD154 + IL-4 stimulation in case #3058, days 3-10.
- Overlay of FSC histogram showing the increase in CLL cell size upon CD154 + IL-4 stimulation in case #3058, days 3-10.
- Forward Scatter (FSC) median raw data average \pm standard deviation. Student paired two-tailed t-test were calculated. * refers to $P < 0.05$, ** refers to $P < 0.01$.
- Normalised Forward Scatter (FSC) median raw data average \pm standard deviation.

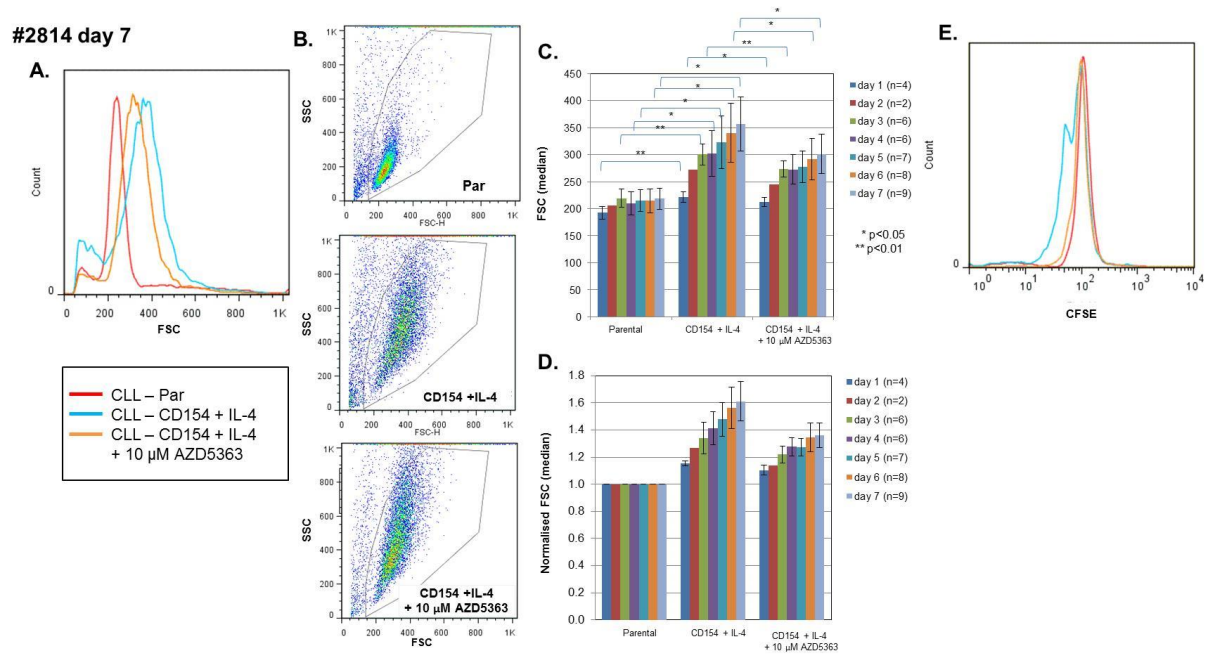


Figure 4.5: Effect of AZD5363 on CD40-induced increase of size of CLL cells.

- A.** FSC histogram showing the increase in CLL cell size upon CD154 + IL-4 stimulation is inhibited by AZD5363 (10 μ M)
- B.** FSC-SSC flow cytometry plots show the increase in cell size upon CD154 + IL-4 stimulation that is inhibited by AZD5363 (10 μ M)
- C.** Forward Scatter (FSC) median raw data average \pm standard deviation. Student paired two-tailed t-test were calculated. * refers to $P < 0.05$, ** refers to $P < 0.01$.
- D.** Normalised Forward Scatter (FSC) median raw data average \pm standard deviation.
- E.** CFSE stained #2814 cells from day 7, shows proliferation occurring.

4.3 AZD5363 does not inhibit proliferation of normal B cells induced by CD40 stimulation + IL-4.

To determine if the anti-proliferative effect of AZD5363 was selective to CLL cells, the experiment was repeated using normal B cells, which were purified from PBMCs obtained from buffy coats using a CLL B-cell selection kit. As shown in Figure 4.6A-C, normal B cells were readily induced to proliferate after four days when co-cultured with CD154-expressing fibroblasts + IL-4, as indicated by the formation of a lower intensity peak on the CFSE fluorescence histogram.

In contrast to CLL cells, proliferation of normal B cells induced by CD40 + IL-4 was not significantly inhibited by treatment with 10 μ M AZD5363 (Figure 4.7A-E). These results indicate that the anti-proliferative effect of AZD5363 is relatively selective to CLL cells. Again, the drug did not induce any significant cell death as measured by PI/flow cytometry method (Figure 4.7F&G).

The increase in size of normal B cells was again observed in normal B cells upon CD154 + IL-4 stimulation, from day 1 to day 4, similar to that seen in CLL cells (Figure 4.8A&B) (day 4, $p=0.07$). However, the increase in the cell size (Figure 4.8A&B) was not significantly inhibited by 10 μ M AZD5363 to a significant level ($p=0.51$, on the fourth day). This may be because of the small sample size of normal B cells studied.

Taken together, the above results showed that stimulation by CD40 + IL-4 also induced proliferation of normal B cells. However, in contrast to that in CLL cells, inhibition of AKT by AZD5363 did not significantly inhibit proliferation, nor did it inhibit the increase in size of normal B cells in response to CD40 + IL-4 stimulation.

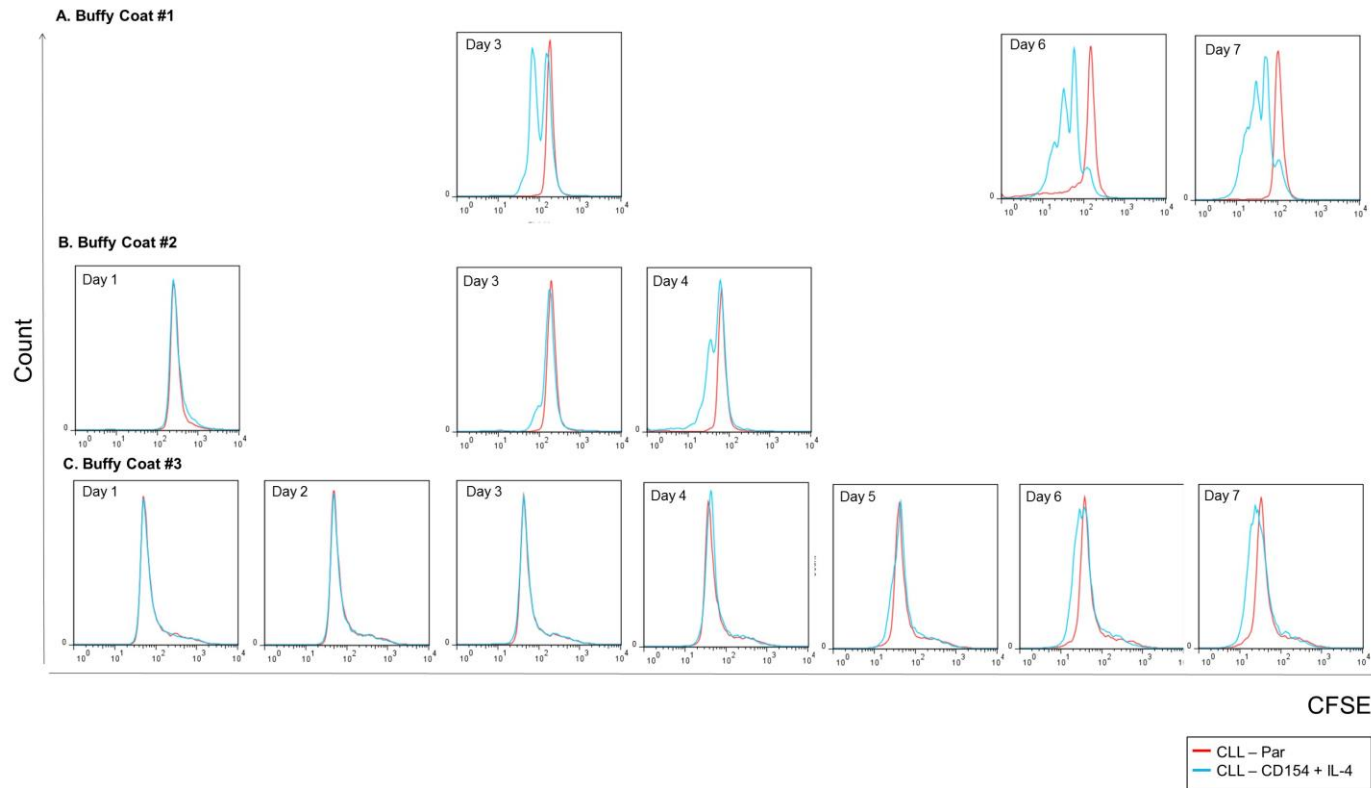
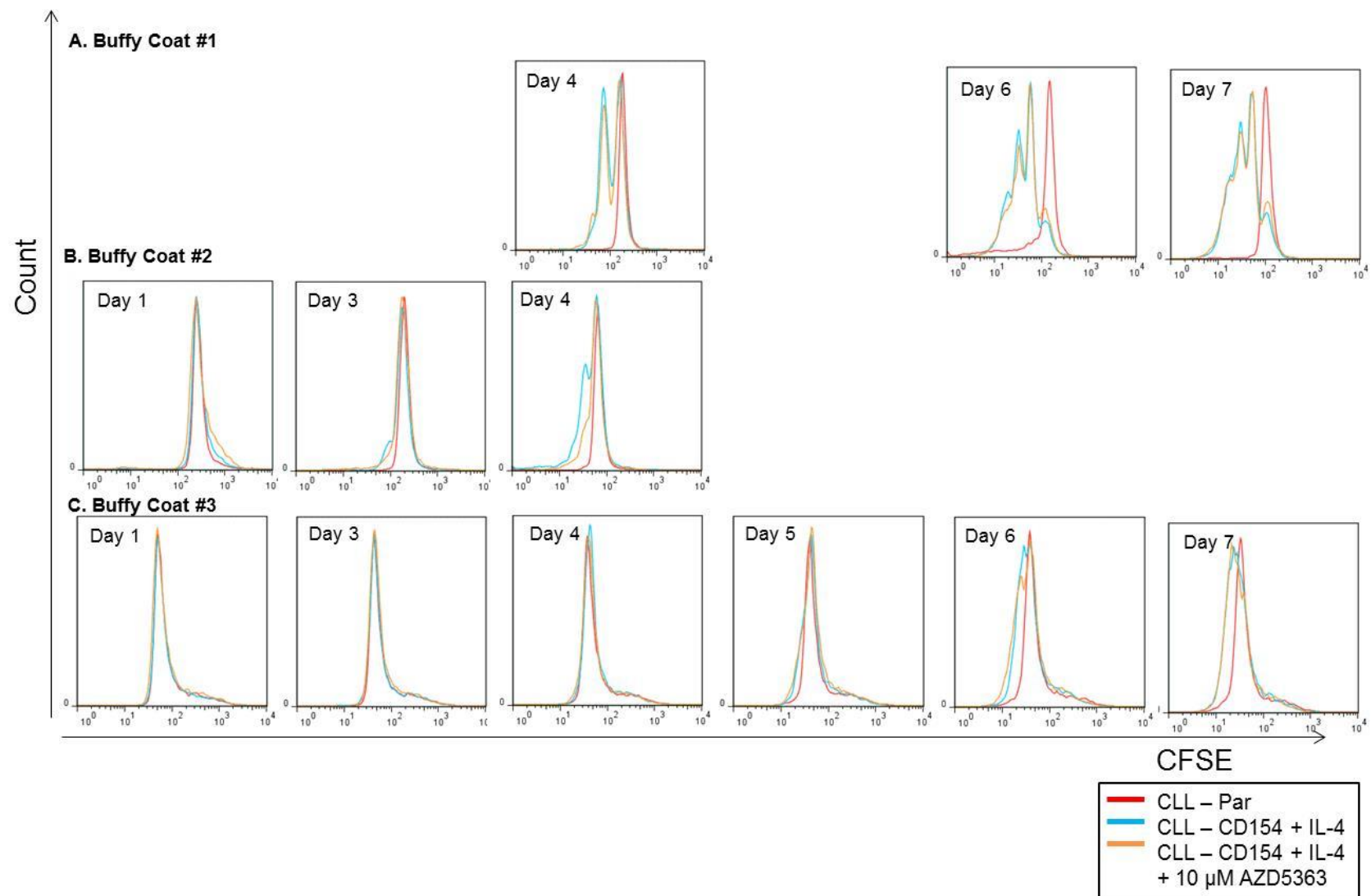


Figure 4.6: CD154 + IL-4 induced B cell proliferation

- A. Normal B cells were purified from buffy coat #1 as described in methods (X), and induced to proliferate by co-culturing them with CD154+ fibroblasts in the presence of human recombinant IL-4. Proliferation was detected by flow cytometry as loss of fluorescence intensity in the histograms as described in the Methods. Normal B cells co-cultured with parental fibroblasts were used as a negative control.
- B. As per (A) but with buffy coat #2.
- C. As per (A) but with buffy coat #3.



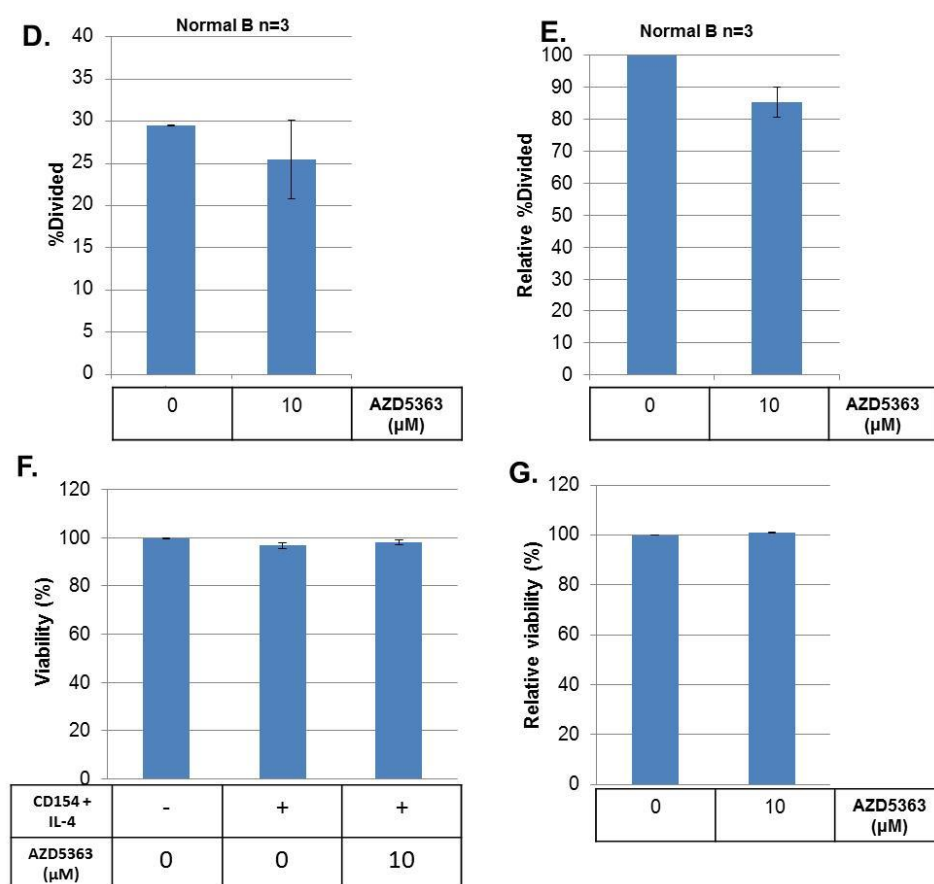


Figure 4.7: CD154 + IL-4 induced normal B-cell proliferation was not significantly inhibited by AZD5363

- A.** Normal B cells were purified from buffy coat #1 as described in methods (X), and induced to proliferate by co-culturing them with CD154+ fibroblasts in the presence of human recombinant IL-4. Proliferation was detected by flow cytometry as loss of fluorescence intensity in the histograms as described in the Methods. Normal B cells co-cultured with parental fibroblasts were used as a negative control. Experiments were performed in the presence or absence of 10 μM AZD5363.
- B.** As per (A) but with buffy coat #2.
- C.** As per (A) but with buffy coat #3.
- D.** Normal B cells (n=3) were stimulated by CD154 + IL-4 for four to seven days and analysed for the percentage divided. When cells were considered to have divided greater than 20%, so day 4 for buffy coat #1 and #2 and day 6 for buffy coat #3, n=3 Average ± Standard deviation % Divided was calculated. The respective values obtained in the absence and presence of 10 μM AZD5363 were 34.4% vs. 31.6% (buffy coat #1 day 4), 25.1% vs. 17.1% (buffy coat #2 day 4), and 28.9% vs. 27.2% (buffy coat #3 day 6). Overall, CD154 +IL-4 stimulated CLL cells were induced to divide 29.5 ± 4.7 % and those with 10 μM AZD5363 divided 25.5 ± 7.2%. Student paired two-tailed t-test were calculated and were non-significant p=0.15.
- E.** In order to see the effect of 10 μM AZD5363, the number of post-mitotic daughter cells upon CD154 +IL-4 stimulation was normalised to 100%, to create a 'relative % divided'. With the addition of 10 μM AZD5363 cells divided 85.4 ± 13.3% compared to that of the control. Student paired two-tailed t-test were calculated and were non-significant p=0.20.
- F.** Cell viability after CD154 + IL-4 induced B-cell proliferation and drug treatment. The % viability of the cells n=3 average (± Standard deviation) was calculated and is presented here.
- G.** Cell viability after CD154 + IL-4 induced B-cell proliferation and drug treatment. The relative % viability n=3 average of the cells was (± Standard deviation) was calculated.

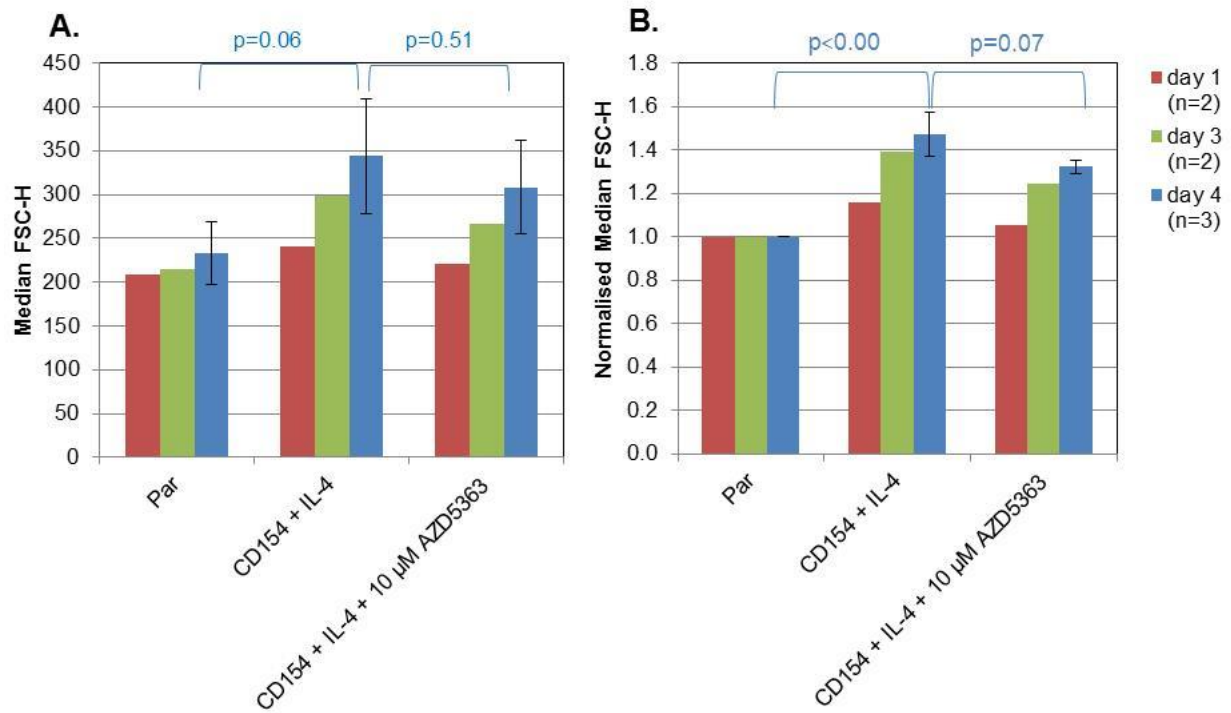


Figure 4.8: Effect of AZD5363 on CD154 + IL-4 induced increase in size of normal B cells

- A.** Normal B cells were purified from buffy coat #1 as described in methods (X), and induced to proliferate by co-culturing them with CD154+ fibroblasts in the presence of human recombinant IL-4. Median Forward Scatter (FSC) raw data from day 4, average $n=3 \pm$ standard deviation. Student paired two-tailed t-test were calculated.
- B.** Normal B cells were purified from buffy coat #1 as described in methods (X), and induced to proliferate by co-culturing them with CD154+ fibroblasts in the presence of human recombinant IL-4. Median Forward Scatter (FSC) data normalised to Normal B cells plated upon parental control cells from day 4, average $n=3 \pm$ standard deviation. Student paired two-tailed t-test were calculated.

4.4 Stimulation by CD154 + IL-21 is more potent in inducing CLL-cell proliferation than CD154 + IL-4

I have shown that CD40 + IL-4 stimulation induced proliferation of CLL cells and this proliferation were selectively inhibited by AZD5363. During the course of these experiments, a paper was published showing that co-culture of CLL cells with CD154-expressing fibroblasts + IL-21 potently induced CLL-cell proliferation (Pascutti et al., 2013).

I therefore sought to determine whether CD40+IL-21 stimulation was more potent than CD40 + IL-4 in inducing CLL-cell proliferation. I used CLL cells from five different samples (#3091, #3106, #3365, #2814 and #3355) and co-cultured them with CD154-expressing fibroblasts in the presence of human recombinant IL-4 (10 ng/ml) (orange-line) or IL-21 (15 ng/ml) (blue-line) (Figure 4.9). Proliferation was measured by loss of CFSE fluorescence intensity on flow cytometry as described previously. CLL cells co-cultured with parental fibroblasts were used as a negative control (red-line) (Figure 4.9).

Quantitative analysis of the above results showed that stimulation with CD40 + IL-21 appeared to be more potent in inducing CLL-cell proliferation than CD40 + IL-4 (Figure 4.10). On day 7, while not all CLL samples had not been induced to proliferate by CD40 + IL-4 stimulation, all CLL samples from different patients examined had been induced to proliferate by CD40 + IL-21 stimulation, albeit to various degrees (Figure 4.10B). On average, CD40 + IL-21 stimulation had induced $35.2 \pm 17.7\%$ proliferation this compares with $8.9 \pm 14.2\%$ proliferation induced by CD154 + IL-4 stimulation by day 7 (Figure 4.10C). It is noteworthy, that even by day 9 and 10, CLL cells from samples #3091 and #3355, had not been induced to proliferate in response to CD40 + IL-4 stimulation (Figure 4.9A&E).

As shown in Figure 4.10D, the time-course experiments comparing the induction of proliferation by two different stimuli clearly showed that CD40 + IL-21 was more potent than CD40 + IL-4 in inducing proliferation of CLL cells. Compared to CD40 + IL-4, CD40 + IL-21 consistently induced significantly higher percentage of divided cells, beginning as early as day 5 and thereafter during the time-course of the experiments (Figure 4.10D).

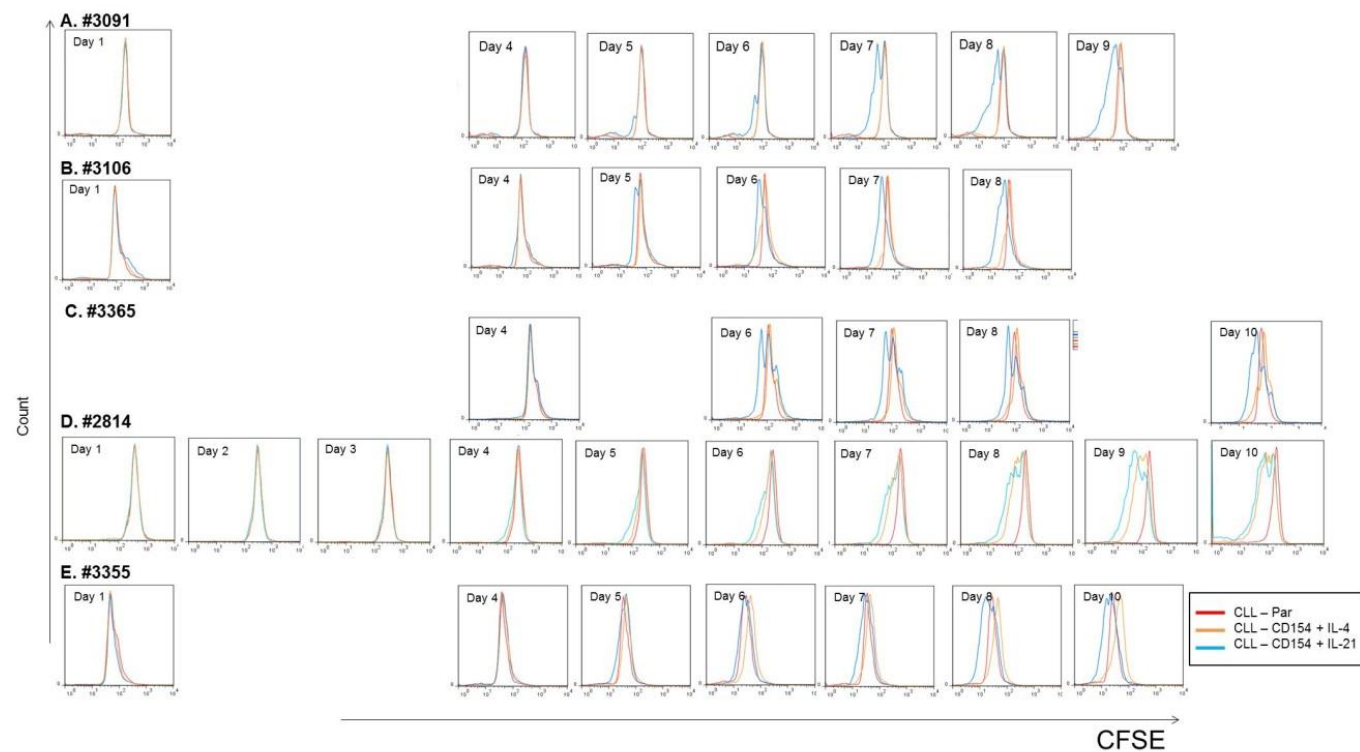


Figure 4.9: Time-dependent induction of proliferation by CD154 + IL-4 versus CD154 + IL-21 in co-cultured CLL cells.

CFSE-stained primary CLL cells were co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-4 (10 ng/ml) (orange-line) or IL-21 (15 ng/ml) (blue-line). Proliferation was detected by flow cytometry as loss of fluorescence intensity as described in the Methods. CLL cells co-cultured with parental fibroblasts were used as a negative control (red-line).

- A. Case #3091.
- B. Case #3106.
- C. Case #3365.
- D. Case #2814.
- E. Case #3355

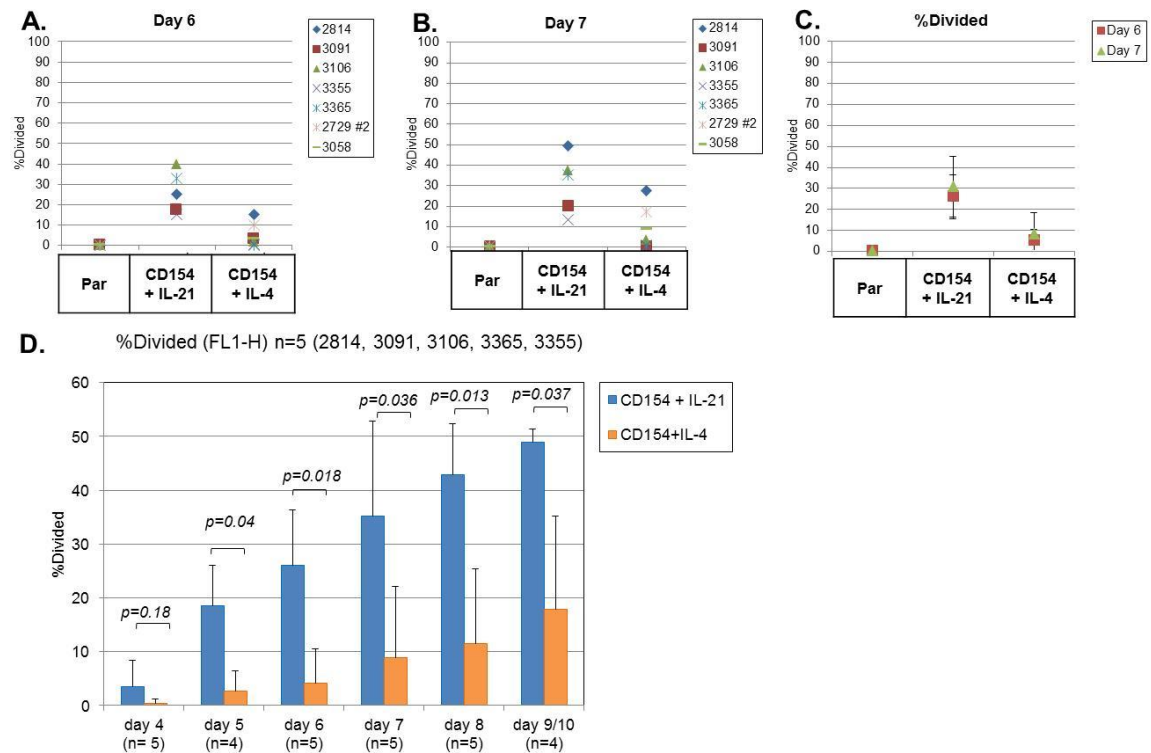


Figure 4.10: CD154 + IL-21 stimulation is a more potent inducer of CLL-cell proliferation than CD154 + IL-4.

CFSE-stained primary CLL cells were co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-4 (10 ng/ml) or IL-21 (15 ng/ml). Proliferation was detected by flow cytometry as loss of fluorescence intensity as described in the Methods. CLL cells co-cultured with parental fibroblasts were used as a negative control. % Divided was calculated using FlowJo.

- A. Individual cases on day 6
- B. Individual cases on day 7
- C. Day 6 and Day 7 n=5 \pm standard deviation
- D. Days 4-10 n number indicated average \pm standard deviation. Student paired two-tailed t-test were calculated.

4.5 AKT is activated by stimulation with CD40 + IL-21.

As described in [section 4.2.2](#), proliferation induced by CD40 + IL-4 involved AKT and inhibition of AKT activity by AZD5363 reduced CD40 + IL-4-induced proliferation. I therefore wanted to know if AKT was also involved in proliferation induced by CD40 + IL-21. I first examined by Western blotting the phosphorylation status of AKT in CLL cells co-cultured with CD154-expressing fibroblasts in the presence of IL-21. IL-4 was also used in the experiments in parallel as a control. I used CLL cells from 4 samples (#3355, #3365, #3129, and #3308) in the experiments and observed that AKT was phosphorylated in CLL cells co-cultured with CD154-expressing fibroblasts (Figure 4.11A-D,E&H). Addition of IL-4 or IL-21 did not appear to significantly increase extent of phosphorylation of AKT (Figure 4.11E&H).

To confirm the activity of IL-21 in these cells, I looked for presence of phospho-STAT3 by Western blotting as a measure of IL-21 activity as reported (Pascutti et al., 2013). As shown in Figure 4.12A&B, CLL cells co-cultured with CD154-expressing fibroblasts in the presence of IL-21 expressed significantly higher level of phospho-STAT3 whereas cells co-cultured with CD154-expressing fibroblasts in the presence of IL-4 did not show such an increase. This confirmed that IL-21 was active in co-cultured CLL cells and that phosphorylation of STAT3 was specific to IL-21.

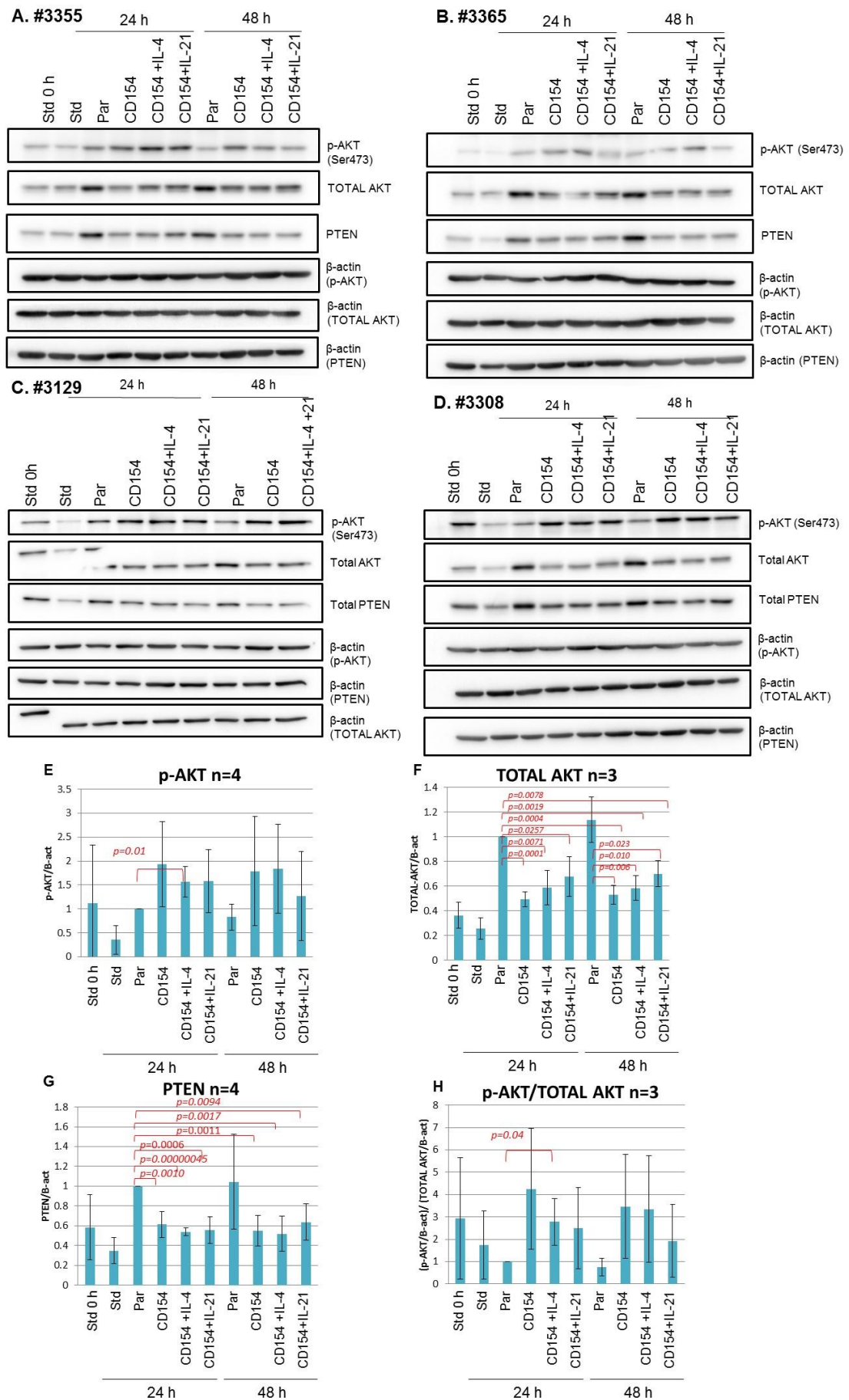


Figure 4.11: AKT is activated in CLL cells stimulated with CD154 with or without IL-4 or IL-21.

CLL cells from four cases were thawed and recovered for 1 hour (Standard culture 0 h, Std 0 h), and cultured for 24 hours either alone (Standard culture 24 h, Std 24 h), or co-cultured on parental fibroblasts (Par) or co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-4 (10 ng/ml) (CD154 + IL-4) or IL-21 (15 ng/ml) (CD154 + IL-21) for 24 hours (24h) or 48 hours (48h). Cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in modified RIPA with sonication, protein determination performed and 10 µg of cell lysate loaded onto 10% SDS-PAGE gel, proteins were transferred onto PVDF membrane. The membrane was incubated in primary antibodies overnight, followed by relevant HRP-conjugated secondary antibodies and the western was developed using ECL on a CCD camera. Densitometry was performed using AIDA image analyser.

A. Western blot of CLL case #3355.

B. Western blot of CLL case #3365.

C. Western blot of CLL case #3129. (Please note: Total AKT blot ripped, hence Total AKT and β-actin from the blot unquantifiable).

D. Western blot of CLL case #3308.

E. Densitometry of p-AKT n= 4.

Densitometry from the four westerns was normalised to Parental 24 hours (Par 24h).
Student paired two-tailed t-test was performed on the data, p-values less than 0.05 are displayed.

F. Densitometry of TOTAL AKT n= 3.

Densitometry from the three westerns was normalised to Parental 24 hours (Par 24h).
#3129 not included, due to TOTAL AKT blot tearing.
Student paired two-tailed t-test was performed on the data, p-values less than 0.05 are displayed.

G. Densitometry of PTEN n= 4.

Densitometry from the four westerns was normalised to Parental 24 hours (Par 24h).
Student paired two-tailed t-test was performed on the data, p-values less than 0.05 are displayed.

H. Densitometry of p-AKT/TOTAL AKT n= 3.

Densitometry from p-AKT was divided by densitometry from TOTAL AKT.
#3129 not included.
Student paired two-tailed t-test was performed on the data, p-values less than 0.05 are displayed.

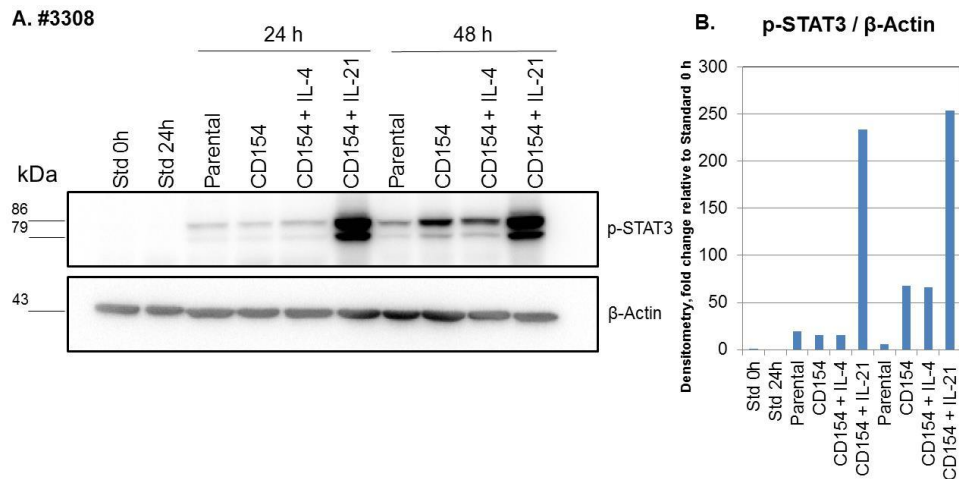


Figure 4.12: CD154 + IL-21 activates STAT3 in CLL cells

CLL case #3308 was thawed and recovered for 1 hour (Standard culture 0 h, Std 0 h), and cultured for 24 hours either alone (Standard culture 24 h, Std 24 h), or co-cultured on parental fibroblasts (Par) or co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-4 (10 ng/ml) (CD154 + IL-4) or IL-21 (15 ng/ml) (CD154 + IL-21) for 24 hours (24h) or 48 hours (48h). Cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in modified RIPA with sonication, protein determination performed and 10 µg of cell lysate loaded onto 10% SDS-PAGE gel, proteins were transferred onto PVDF membrane. The membrane was incubated in primary antibodies overnight, followed by relevant HRP-conjugated secondary antibodies and the western was developed using ECL on a CCD camera. Densitometry was performed using AIDA image analyser.

A. Western blot of CLL case #3308

B. Densitometry of p-STAT3 / β-actin

4.6 The effect of AKT inhibitors upon CD40 + IL-21-induced CLL proliferation.

I have shown earlier that AKT was required for proliferation induced by CD40 + IL-4 stimulation in CLL cells as AKT inhibitor AZD5363 inhibited this proliferation ([section 4.2](#)). In addition, I have also shown in the previous section that AKT was activated in CLL cells stimulated with CD40 + IL-21. In light of CD40 + IL-21 stimulation being a more potent inducer of proliferation ([section 4.4](#), Figure 4.9 and Figure 4.10), I wanted to determine if AKT is also required in CLL-cell proliferation induced by CD40 + IL-21 stimulation. To do this, I used AKT inhibitor AZD5363 to test if inhibition of AKT will also inhibit CD40 + IL-21-induced proliferation using primary CLL cells from five different patients' samples.

As shown in Figure 4.13A-C, AZD5363 inhibited such proliferation in a dose-dependent manner in CLL cells co-cultured with CD154-expressing fibroblasts in the presence of IL-21 from three patients samples (#3091, #3106 and #3365) (Figure 4.14A). However, the AKT inhibitor did not inhibit the CD40 + IL-21-induced

proliferation in CLL cells from two other samples (#3355 and #2814) (Figure 4.13D&E, Figure 4.14B&C). AZD5363 did not affect the cell viability at the concentrations used (1 – 10 μ M) (Figure 4.15A). The above result was in contradiction to the previous finding that AZD5363 (10 μ M) inhibited the CD40 + IL-4-induced proliferation in CLL cells from all the samples examined (Figure 4.2 and Figure 4.3). This suggested that AKT was required for CD40 + IL-21-induced proliferation in some, but not all, CLL samples.

To confirm the specificity of AZD5363, I used another AKT inhibitor in the experiments. I chose MK-2206 as it inhibits AKT activity by binding to the plextrin-homology (PH) domain of the enzyme and preventing its translocation to the membrane and activation (Hirai et al., 2010). MK-2206 has also been shown to induce apoptosis of CLL cells *in vitro*, with 8 μ M concentration of the drug able to kill 50% of CLL cells after 72 hours of incubation (Ding et al., 2013). I therefore used 1, 3 and 10 μ M MK-2206 in my experiments.

Similar to AZD5363, MK-2206 inhibited CD40 + IL-21-induced CLL-cell proliferation in the same three CLL samples (#3091, #3106 and #3365) (Figure 4.13F-H, Figure 4.14D). Again, MK-2206 also failed to inhibit such proliferation in two other samples (#2814 and #3355) (Figure 4.13I&J, Figure 4.14E&F). MK-2206 did not affect the cell viability at 1 and 3 μ M. At 10 μ M MK-2206, around 30% cell death occurred on days 6-8 (Figure 4.15B).

Therefore, two AKT inhibitors (AZD5363 and MK-2206) with different modes of action in inhibiting AKT activity produced consistent effects: both inhibiting CD40 + IL-21-induced proliferation in CLL cells from the same three patient samples but failing to inhibiting such proliferation in CLL cells from two other samples. This suggested that AKT was required for CD40 + IL-21-induced proliferation in some but not all CLL cells.

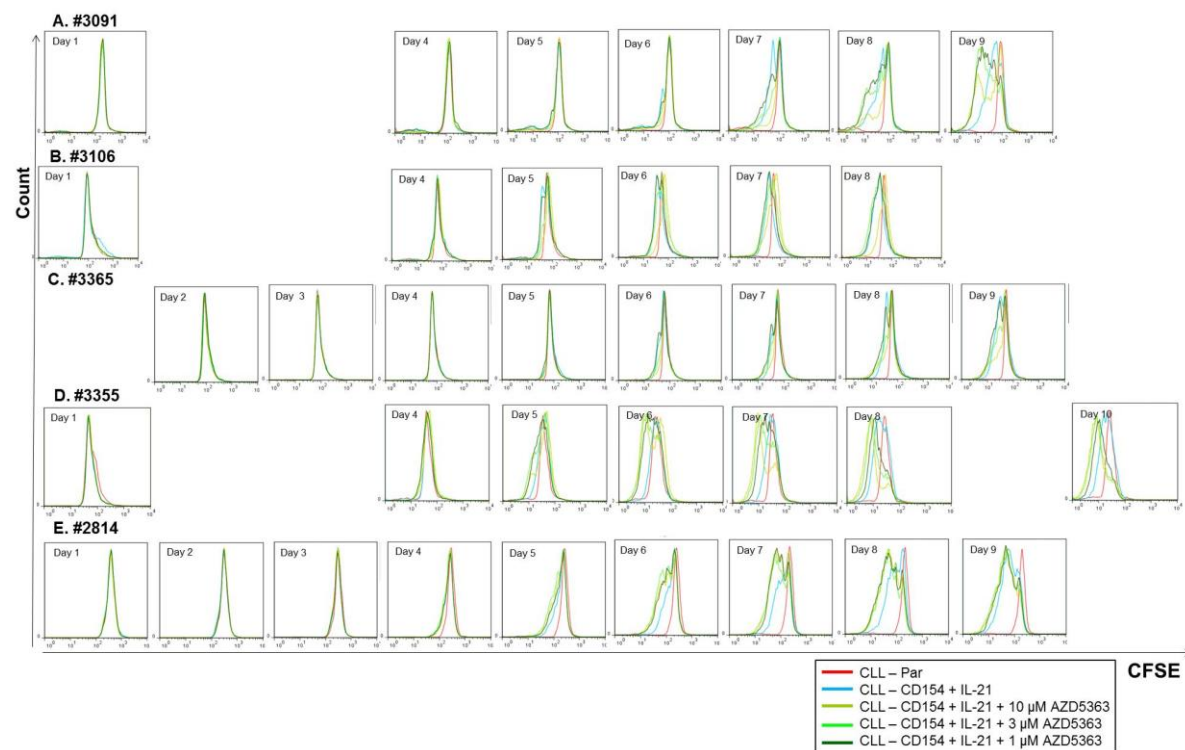


Figure 4.13: The effect of AKT inhibitors on CD154 + IL-21 induced CLL proliferation.

CFSE-stained primary CLL cells were co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-21 (15 ng/ml) (CD154 + IL-21) and AKT inhibitors at the indicated concentrations. Media was changed on day 3 and day 6. Proliferation was detected by flow cytometry as loss of fluorescence intensity as described in the Methods. CLL cells co-cultured with parental fibroblasts (Par) were used as a negative control.

- A. #3091 AKT kinase-domain inhibitor: AZD5363.
- B. #3106 AKT kinase-domain inhibitor: AZD5363.
- C. #3365 AKT kinase-domain inhibitor: AZD5363.
- D. #3355 AKT kinase-domain inhibitor: AZD5363.
- E. #2814 AKT kinase-domain inhibitor: AZD5363.

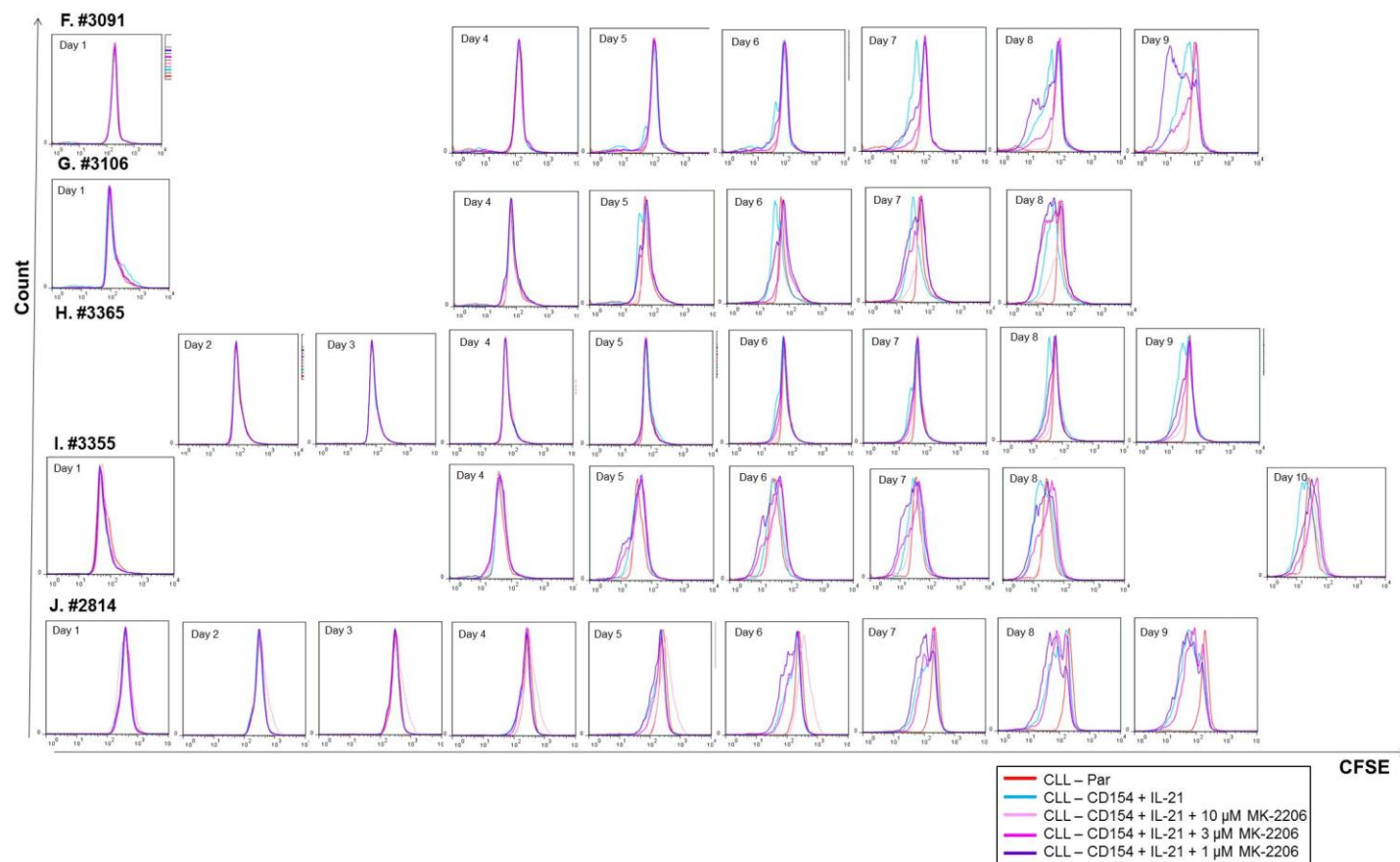
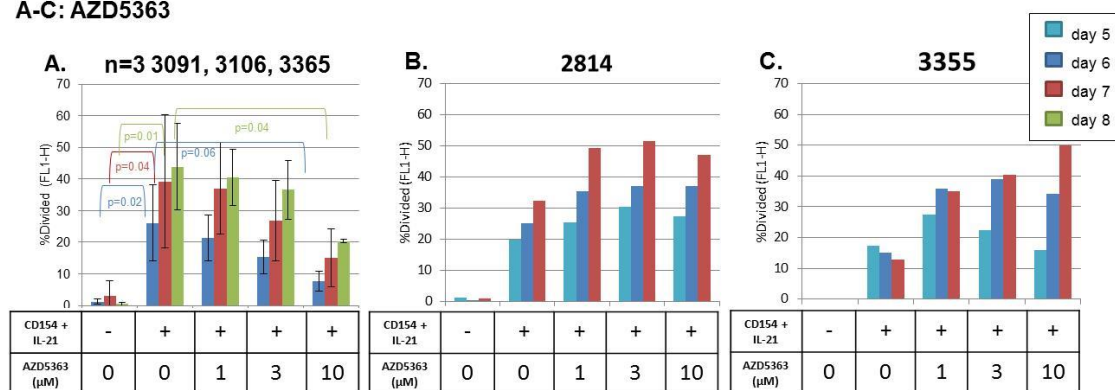


Figure 4.13: The effect of AKT inhibitors on CD154 + IL-21 induced CLL proliferation (continued).

- F. #3091 AKT PH-domain inhibitor: MK-2206.
 G. #3106 AKT PH-domain inhibitor: MK-2206.
 H. #3365 AKT PH-domain inhibitor: MK-2206.
 I. #3355 AKT PH-domain inhibitor: MK-2206.
 J. #2814 AKT PH-domain inhibitor: MK-2206

A-C: AZD5363



D-F: MK-2206

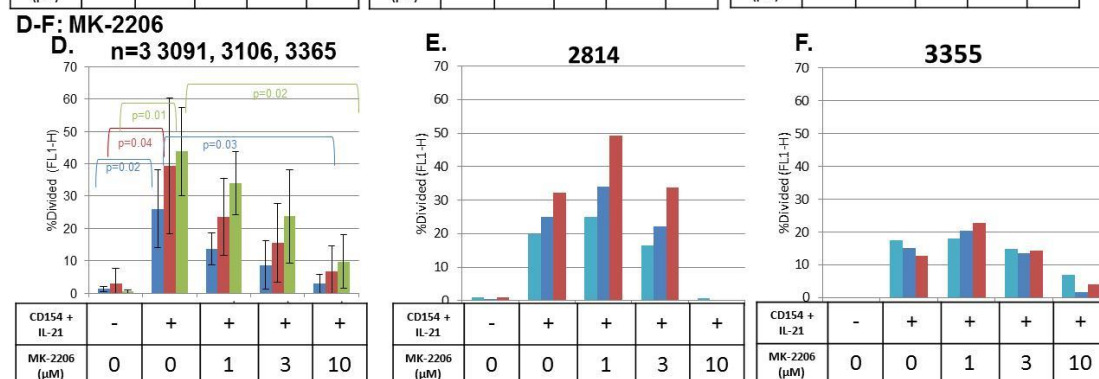


Figure 4.14: Quantitative analysis of the effect of AKT inhibitors upon CLL-cell proliferation induced by the CD154 + IL-21 stimulation.

CFSE-stained primary CLL cells were co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-21 (15 ng/ml) (CD154 + IL-21) and AKT inhibitors at the indicated concentrations. Media was changed on day 3 and day 6. Live cell viability was measured using propidium iodide (PI) intensity as described in the Methods. CLL cells co-cultured with parental fibroblasts (Par) were used as a negative control. % Divided was calculated for five CLL cases induced to proliferate with CD154 + IL-21. Three cases (#3091, #3106, #3365) clearly showed inhibition of proliferation upon addition of the AKT inhibitors. Two cases #2814, #3355, proliferation was not inhibited by AKT inhibitors. As such, the data is presented separately. % Divided data is presented for day 5, day 6, day 7 and day 8.

A-C: AKT kinase-domain inhibitor: AZD5363.

- A. Average of n=3 #3091, #3106, #3365 (± Standard deviation). Student paired two-tailed t-test was performed on the data, p-values less than 0.05 are displayed.
- B. #2814.
- C. #3355.

D-F: AKT PH-domain inhibitor: MK-2206.

- D. Average of n=3 #3091, #3106, #3365 (± Standard deviation). Student paired two-tailed t-test was performed on the data, p-values less than 0.05 are displayed.
- E. #2814.
- F. #3355.

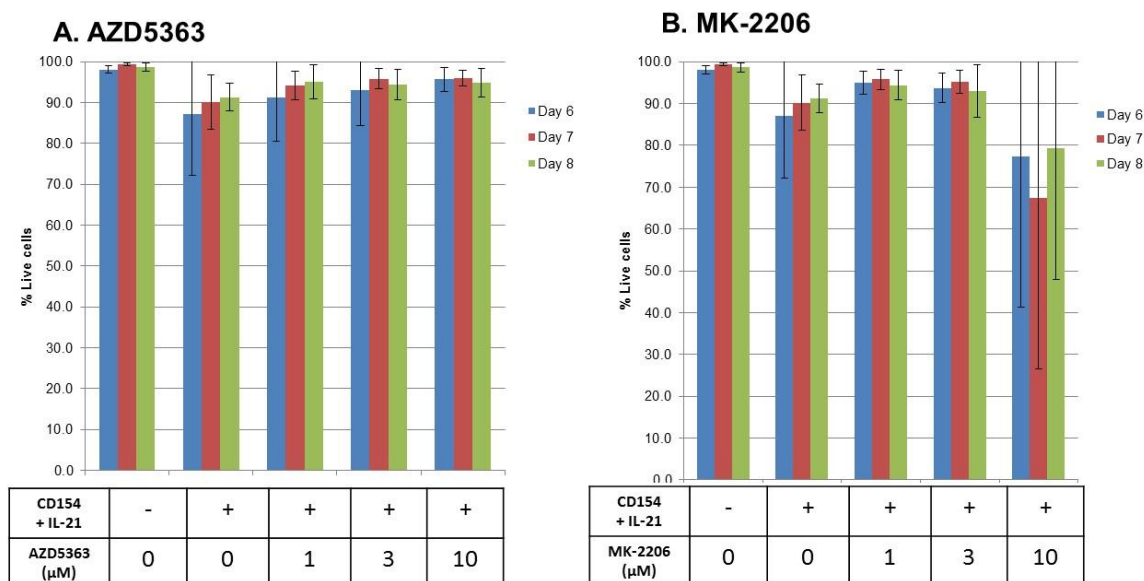


Figure 4.15: The effect of AKT inhibitors upon cell viability of CLL cells stimulated by CD154 + IL-21.

CFSE-stained primary CLL cells were co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-21 (15 ng/ml) and AKT inhibitors at the indicated concentrations. Media was changed on day 3 and day 6. Live cell viability was measured using propidium iodide (PI) intensity as described in the Methods. CLL cells co-cultured with parental fibroblasts were used as a negative control. n=5; 3091, 3106, 3365, 2814, 3355. Student paired two-tailed t-test was performed on the data, p-values less than 0.05 are displayed.

A. AZD5363 (% live cells).

B. MK-2206 (% live cells).

4.7 Effect of AKT inhibition on cell growth (increase in cell size) of CD40 + IL-21-stimulated CLL cells

I previously showed that AZD5363 inhibited cell growth as measured by increase in size of CD40 +IL-4-stimulated CLL cells ([section 4.2.3](#)). Here I also wanted to determine whether AKT inhibition by both AZD5363 and MK-2206 also inhibited CD40 + IL-21-induced cell growth. As shown in Figure 4.16F-I, CLL cells co-cultured with CD154-expressing fibroblasts + IL-21 displayed increased cell size in a time-dependent manner. After only one day of CD40+IL-21 stimulation of CLL cells, their cell size had increased by 1.19 fold comparing to the size of cells prior to stimulation. Cell size increased by 1.35 fold after two days, and by 1.44 fold and 1.55 fold on day 3 and 4, respectively (Figure 4.16H&I). After six days of stimulation with CD40+IL-21, the CLL cells had increased in size by 1.67 fold and this increased further to 1.80 fold after eight days (Figure 4.16H&I).

Interestingly, addition of the AKT inhibitors AZD5363 and MK-2206, consistently inhibited the increase in cell size of CD40 + IL-21-stimulated CLL cells in all samples studied (Figure 4.16A, D&E, F-I). For example, CLL cells stimulated by CD40 +IL-21 for five days increased in cell size by 1.66 fold, however the addition of AZD5363 (10 μ M) or MK-2206 (3 μ M) reduced this increase in size to 1.4 fold. Furthermore, CLL cells stimulated with CD40 + IL-21 for eight days increased the cell size by 1.80 fold. However the cells treated with 1 μ M, 3 μ M and 10 μ M AZD5363 displayed reduced increase in size to 1.66, 1.64 and 1.58 fold respectively, and MK-2206 (3 μ M) also reduced the increase in cell size to 1.50 fold. This suggests that AKT is required in growth of CLL cells that were stimulated by CD40 + IL-21.

A. #3091 day 7

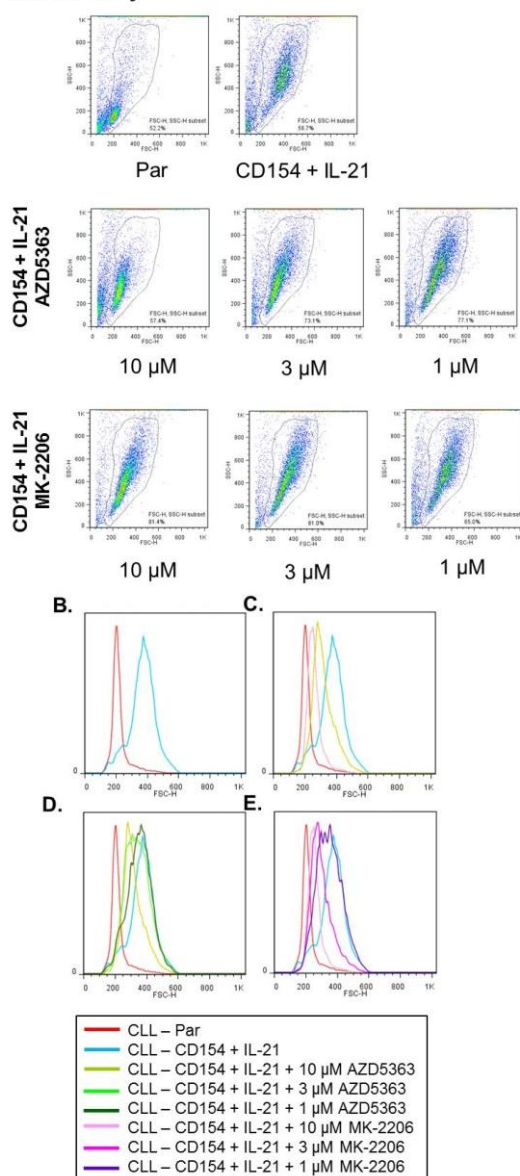


Figure 4.16: Effect of AKT inhibition on size of CLL cells stimulated by CD154 + IL-21.

CLL PBMC cells were thawed out, recovered for 1 hour, prior to CFSE labelling, treating with indicated drug concentrations and then plating out on either parental fibroblasts or CD154 fibroblasts + IL-21 (15 ng/ml) for varying periods of time prior to harvesting. Media including cytokines and drugs was replaced on day 3 and day 6. Median (FSC-H) data is presented here. n=5 Average \pm Standard deviation, days 5-8. p-values were calculated using paired student t-test in excel.

- FSC-SSC plots, #3091 day 7.
- Histograms of FSC, showing the increase in FSC upon CD154 + IL-21 stimulation, #3091 day 7
- The effect of the highest doses of AZD5363 and MK-2206 upon FSC, following CD154 + IL-21 stimulation.
- The effect of AZD5363 upon FSC, following CD154 + IL-21 stimulation.
- The effect of MK-2206 upon FSC, following CD154 + IL-21 stimulation.

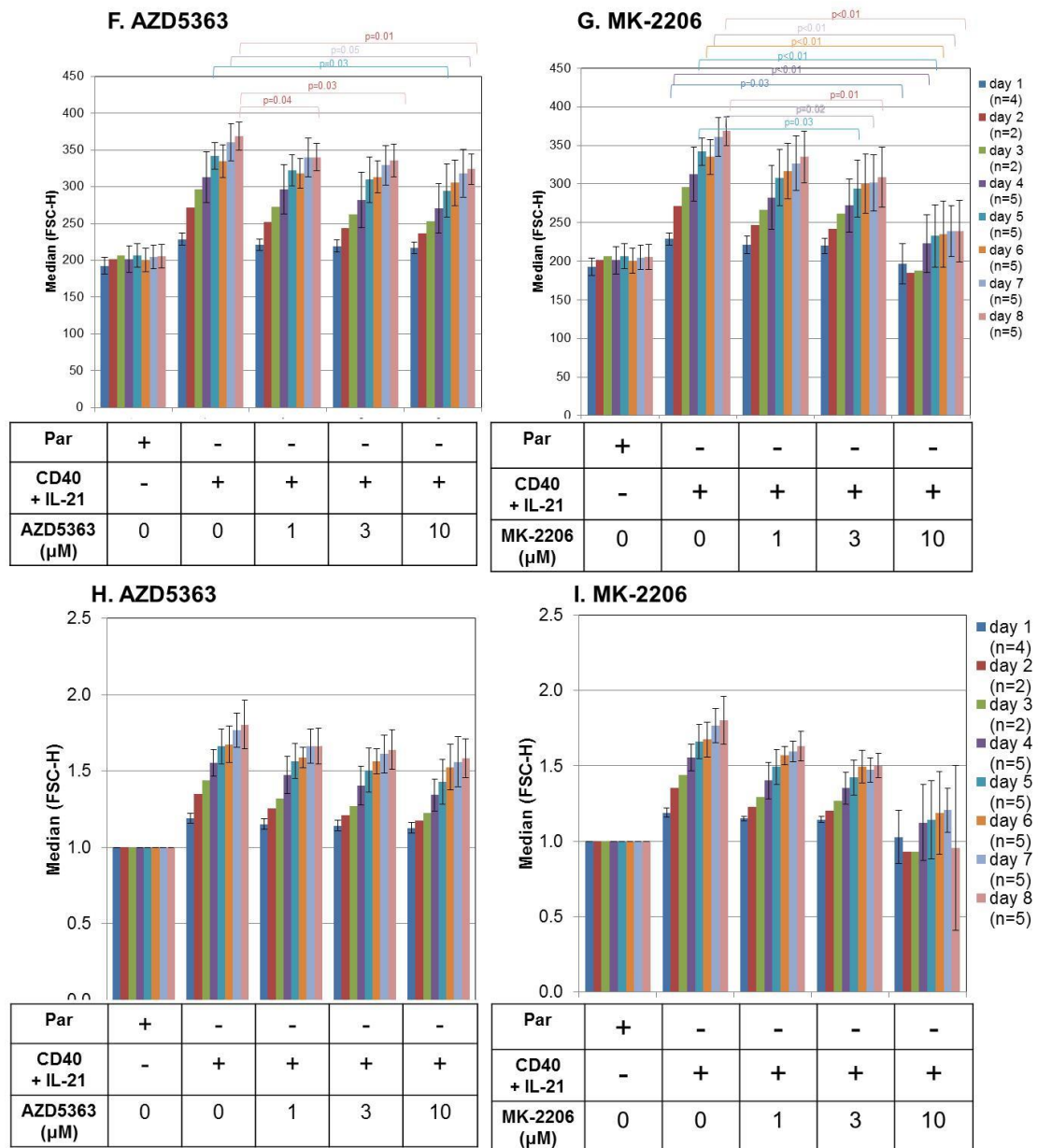


Figure 4.16: Effect of AKT inhibition on size of CLL cells stimulated by CD154 + IL-21 (continued).

- F.** AZD5363, Median (FSC-H). Average value \pm standard deviation, student two-tailed t-tests were performed, p values less than 0.05 are displayed.
- G.** MK-2206, Median (FSC-H). Average value \pm standard deviation, student two-tailed t-tests were performed, p values less than 0.05 are displayed.
- H.** Normalised data: AZD5363, Median (FSC-H).
- I.** Normalised data: MK-2206, Median (FSC-H).

4.8 The divided cells following CD154 + IL-21 stimulation are B-CLL cells.

Finally, to prove that the proliferating cells were really B-CLL cells, I checked the percentage of CD19 positive cells among cell population that displayed reduced CFSE fluorescent intensity upon FACS analysis. I first harvested CFSE-labelled CLL cells that were co-cultured with CD154-expressing fibroblasts + IL-21 for the indicated time periods and incubated these cells with an anti-CD19 antibody before FACS analysis. As shown in Figure 4.17, co-cultured CLL cells (sample #3091) harvested on day 7 had 81.2% cells that were positive for CD19 among the cell population with reduced CFSE fluorescence. Co-cultured CLL cells (sample #3091) harvested on day 8 and 9 had 75.6% and 86.6% cells that were positive for CD19 among the cell population with reduced CFSE fluorescence (data not shown).

To further prove that these were CLL-B cells, I harvested CFSE-stained CLL cells on co-cultures as described earlier and incubated these cells with both anti-CD5 and anti-CD19 antibodies. As shown in Figure 4.18, on day 5, #3106, 81% of cells with reduced CFSE fluorescence (therefore cells that can be considered to be dividing) were double positive for CD5 and CD19. On day 6, 7 and 8, there were respective 88.8%, 81.6% and 84.2% of CLL cells with reduced CFSE fluorescence double positive for CD5 and CD19 (data not shown). Therefore the above results confirmed that the majority of the proliferating cells in response to CD40 + IL-21 stimulation were CLL B cells.

#3091 Day 7

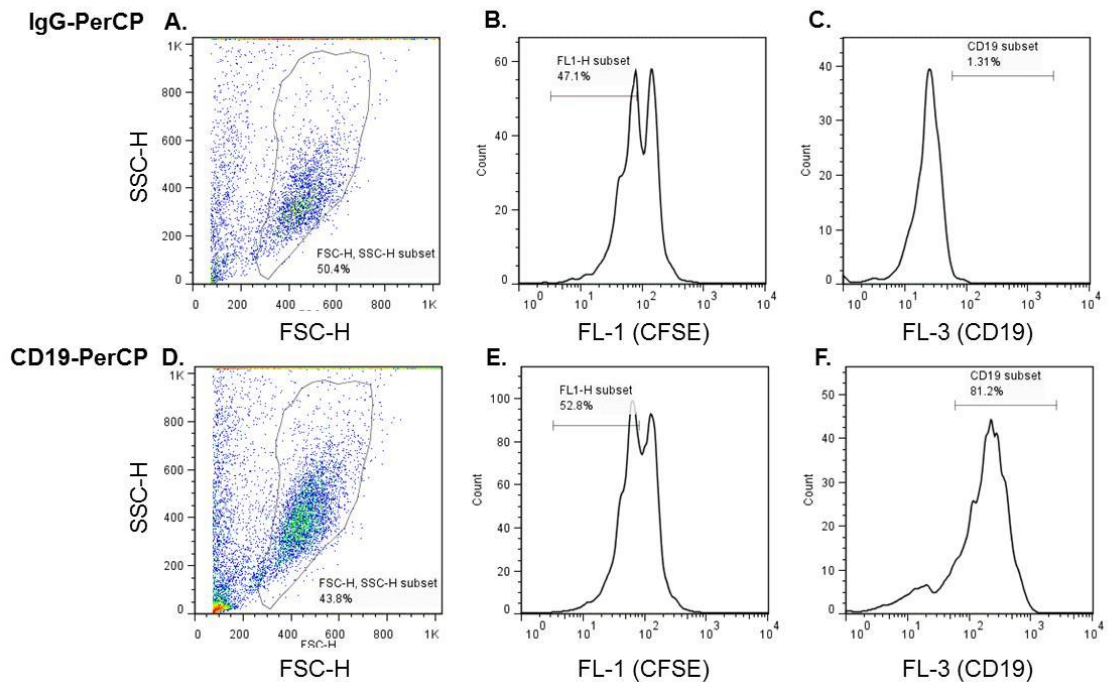


Figure 4.17: The dividing cells induced by CD154 + IL-21 stimulation are CD19⁺ B cells.

CLL PBMC cells were thawed out, recovered for 1 hour, prior to CFSE labelling, and then plating out on CD154 fibroblasts + IL-21 (15 ng/ml) for between seven and nine days prior to harvesting. CLL cells were stained either with control antibody (IgG-PerCP) or CD19-PerCP (see antibodies in methods and materials) and examined via flow cytometry. On day 7:

A/D. Cells were gated to ignore cell debris.

B/E. CFSE staining was examined (FL-1). Cells to the left of the original peak were further examined.

C/F. CD19⁺ cells were examined (FL-3).

In the featured example, #3091 on day 7: 81.2% of dividing cells were CD19⁺. On day 8 & day 9: 75.6% and 86.6% of #3091 dividing cells were CD19⁺. Therefore it really is B cells that are proliferating under CD154 + IL-21 stimulation.

IgG is used as an isotype antibody control. Isotype controls are chosen that matches the host species, isotype and fluorochrome of the primary antibody. Isotype controls are used in flow cytometry to: ensure that the observed staining is due to specific antibody binding rather than an artefact, exclude non-specific binding of the antibody to Fc receptors, and to exclude other non-specific binding of the antibody or fluorochromes to cellular component.

#3106 day 5

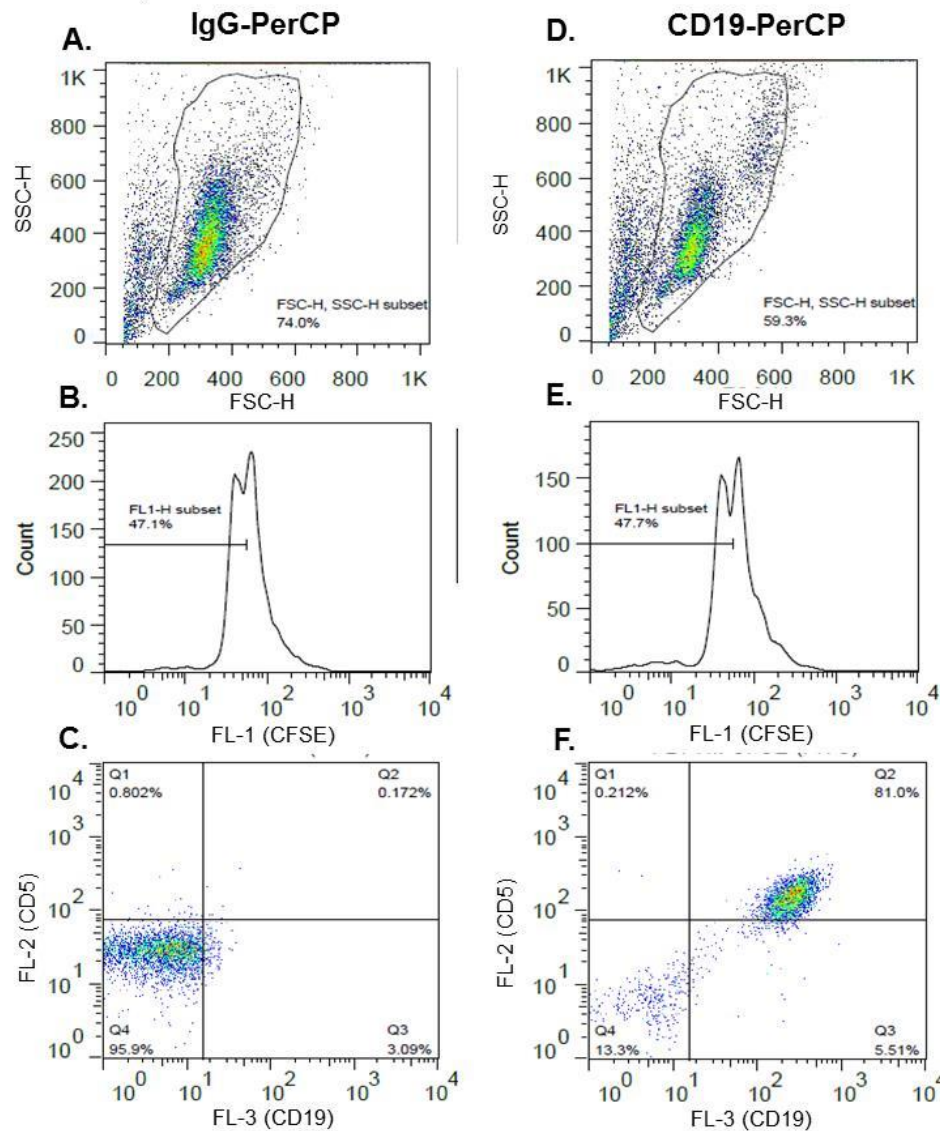


Figure 4.18: The dividing cells under CD154 + IL-21 stimulation are CD5 & CD19 double positive CLL cells.

CLL PBMC cells were thawed out, recovered for 1 hour, prior to CFSE labelling, and then plating out on CD154 fibroblasts + IL-21 (15 ng/ml) for between five and eight days prior to harvesting. CLL cells were stained either with control antibodies (IgG-PE, IgG-PerCP) or CD5-PE, CD19-PerCP (see antibodies in methods and materials) and examined via flow cytometry. On day 5:

A/D. Cells were gated to ignore cell debris.

B/E. CFSE staining was examined (FL-1). Cells to the left of the original peak, were further examined;

C/F. CD5+CD19+ cells were examined (FL-2, FL-3).

In this featured example. #3106, on day 5: 81% of dividing cells were CD5+CD19+. On day 6,7 and 8: 88.8%, 81.6% and 84.2% of #3091 dividing cells were CD5+ CD19+. Therefore it really is CLL B cells that are proliferating under CD154 + IL-21 stimulation.

IgG is used as an isotype antibody control. Isotype controls are chosen that matches the host species, isotype and fluorochrome of the primary antibody. Isotype controls are used in flow cytometry to: ensure that the observed staining is due to specific antibody binding rather than an artefact, exclude non-specific binding of the antibody to Fc receptors, and to exclude other non-specific binding of the antibody or fluorochromes to cellular component.

4.9 Effect of AKT inhibitors on the proliferation of normal B cells induced by CD40 + IL-21 stimulation.

Consistent with results obtained from the CD40 + IL-4 proliferation assay, normal B cells isolated from buffy coats also proliferated faster than CLL cells in the CD40 + IL-21 assay (Figure 4.19). As shown in Figure 4.21A, significant amounts of divided cells ($36.0 \pm 25.1\%$) were detected by day 4 from the normal B cells that were co-cultured with CD154-expressing fibroblasts in the presence of IL-21. By day 5, $57.5 \pm 18.2\%$ of divided cells were observed (Figure 4.21A).

However, neither AZD5363 nor MK-2206 inhibited the proliferation of normal B cells induced by CD40 + IL-21 stimulation (Figure 4.20). As shown in Figure 4.21A&B, AZD5363 at the range of concentrations tested did not reduce the amount of divided cells throughout the time period of observation. If anything, the addition of AZD5363 (1 μ M and 3 μ M) appeared to increase the proliferation of normal B cells on days 3 and 4 (Figure 4.21B), although why this happened remains unclear. Also, addition of MK-2206 had little effect on the CD40 + IL-21-induced proliferation of normal B cells (Figure 4.21B). It is important to note that there was no significant induction of cell death in normal B cells by either AZD5363 or MK-2206 at the concentrations used in the study (Figure 4.21C).

The size of normal B cells, as measured by FSC on flow cytometry, also increased in a time-dependent manner in response to CD40 + IL-21 stimulation. Normal B cells co-cultured with CD154-expressing fibroblasts in the presence of IL-21 increased cell size by 1.79-fold on day 4 and 1.85-fold on day 5, when compared to cells co-cultured with control fibroblasts + IL-21 (Figure 4.21D). However, this increase was also inhibited by the AKT inhibitors. By day 5, AZD5363 at 3 μ M and 10 μ M concentrations significantly reduced the increase in size of CD40 + IL-21-stimulated B cells from 1.85 fold to 1.73 and 1.70-fold, respectively, with their respective p values of 0.02 and 0.03 (Figure 4.21D). Similarly, MK-2206 at 3 μ M significantly reduced the increase in size of CD40 + IL-21-stimulated cells from 1.85 fold to 1.74-fold on day 5 ($p = 0.01$).

Taken together, the above results showed that AKT inhibition by the two inhibitors AZD5363 and MK-2206 did not inhibit the proliferation of normal B cells induced

by CD40 + IL-21 stimulation. However, the two AKT inhibitors significantly reduced the cell growth as measured by increase in size of CD40 + IL-21-stimulated normal B cells. Therefore, these results suggest that AKT is required for the growth but not proliferation of normal B cells in response to CD40 + IL-21 stimulation.

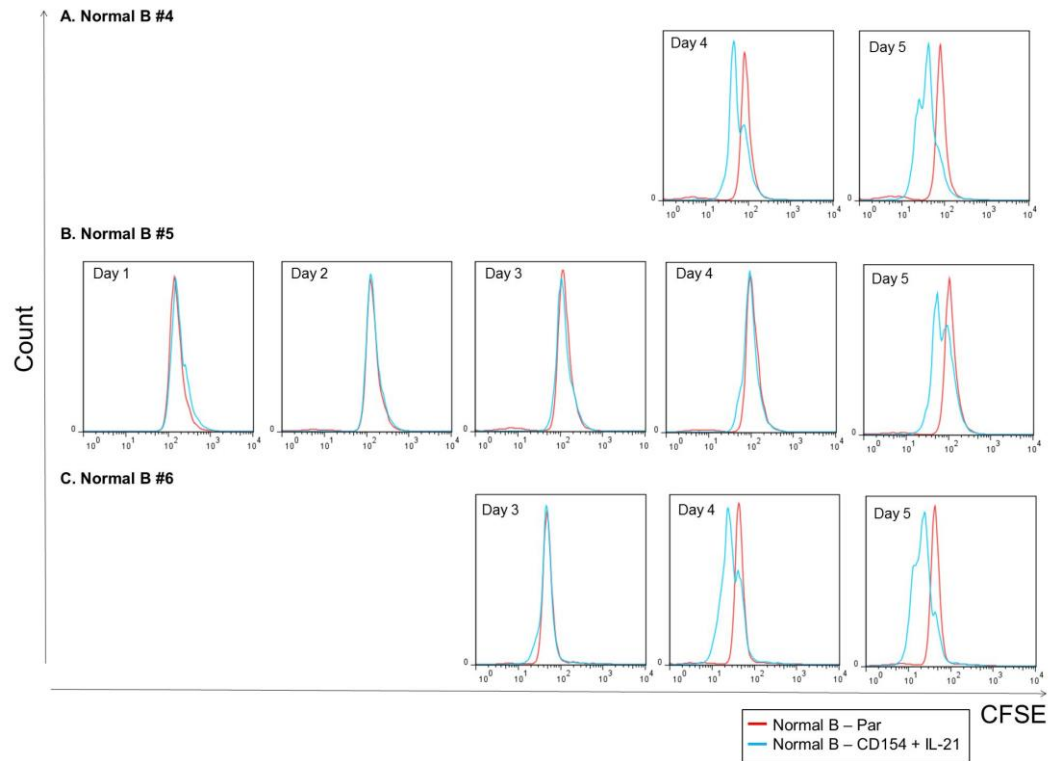


Figure 4.19: The proliferation of normal B cells induced by CD154 + IL-21.

Normal B cells were purified from buffy coats as described in methods (X), CFSE-stained described in methods (X), and were co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-21 (15 ng/ml) (CD154 + IL-21) and AKT inhibitors at the indicated concentrations. Media was changed on day 3 and day 6. Proliferation was detected by flow cytometry as loss of fluorescence intensity as described in the Methods. Normal B cells co-cultured with parental fibroblasts (Par) were used as a negative control.

- A. Buffy coat normal B cells #4.
- B. Buffy coat normal B cells #5.
- C. Buffy coat normal B cells #6.

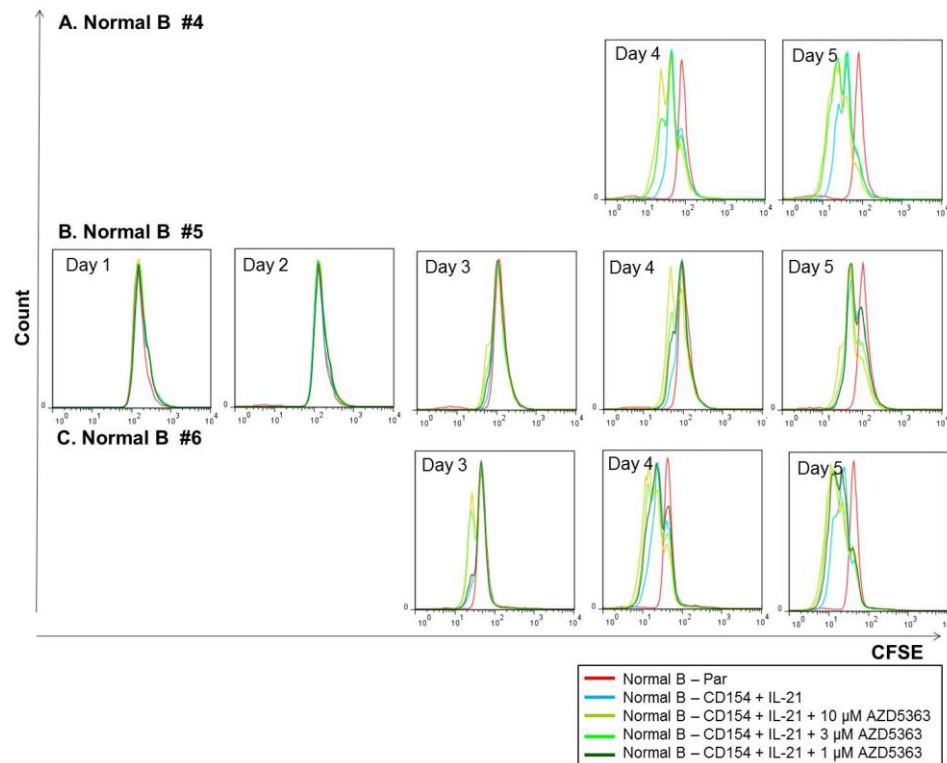


Figure 4.20: Effect of AKT inhibitors on the proliferation of normal B cells induced by CD154 + IL-21.

Normal B cells were purified from buffy coats as described in methods (X), CFSE-stained described in methods (X), and were co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-21 (15 ng/ml) (CD154 + IL-21) and AKT inhibitors at the indicated concentrations. Media was changed on day 3 and day 6. Proliferation was detected by flow cytometry as loss of fluorescence intensity as described in the Methods. Normal B cells co-cultured with parental fibroblasts (Par) were used as a negative control.

- A. Buffy coat normal B cells #4 AKT kinase-domain inhibitor: AZD5363.
- B. Buffy coat normal B cells #5 AKT kinase-domain inhibitor: AZD5363.
- C. Buffy coat normal B cells #6 AKT kinase-domain inhibitor: AZD5363.

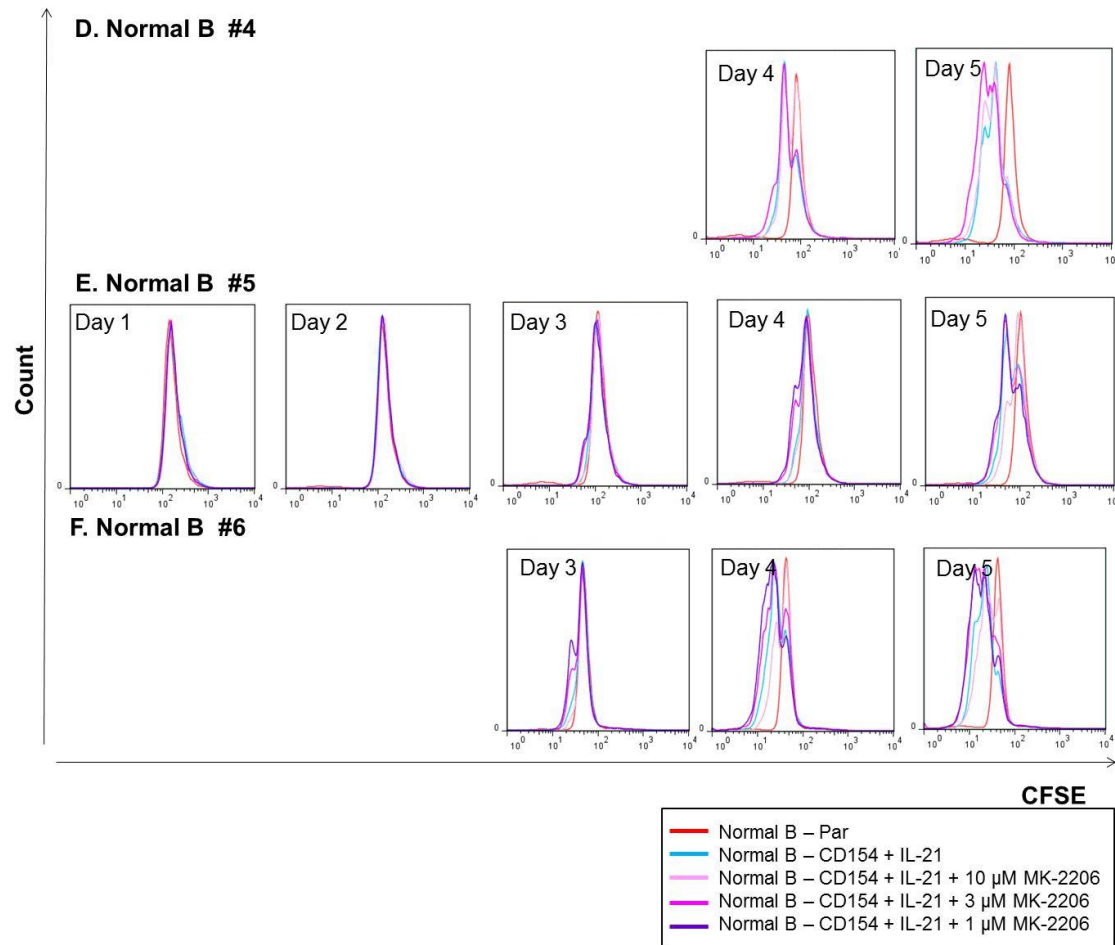


Figure 4.20: Effect of AKT inhibitors on the proliferation of normal B cells induced by CD154 + IL-21 (continued).

- D.** Buffy coat normal B cells #4 AKT PH-domain inhibitor: MK-2206.
E. Buffy coat normal B cells #5 AKT PH-domain inhibitor: MK-2206.
F. Buffy coat normal B cells #6 AKT PH-domain inhibitor: MK-220

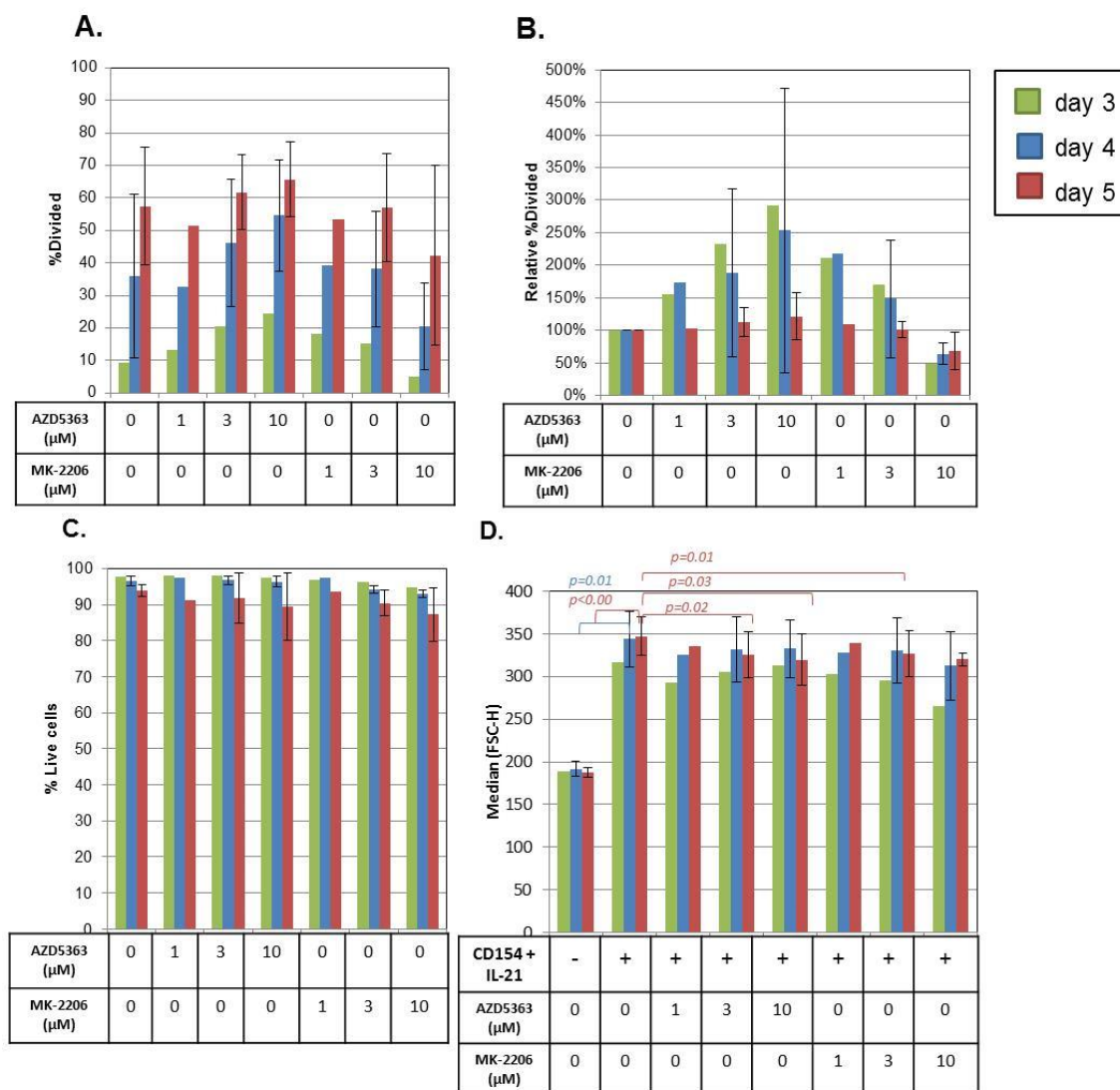


Figure 4.21: Effect of AKT inhibitors on the proliferation and size of normal B cells induced by CD154 + IL-21.

Normal B cells were purified from buffy coats, CFSE-stained and co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-21 (15 ng/ml) (CD154 + IL-21) and AKT inhibitors at the indicated concentrations. Media was changed on day 3. Normal B cells co-cultured with parental fibroblasts (Par) were used as a negative control.

A. % Divided data

% Divided was calculated for three normal B cases induced to proliferate with CD154 + IL-21. Average (n=2) is presented for day 3 and average (n=3) \pm standard deviation for day 4 and day 5. MK-2206 (1 μM) and AZD5363 (1 μM) data points only have n=2, hence no error bars. Student paired two-tailed t-test was performed on the data, p-values less than 0.05 are displayed.

B. Relative % Divided

Data was normalised to CD154 + IL-21 on each day to create a measure 'Relative % Divided'. Average (n=2) is presented for day 3 and average (n=3) \pm standard deviation for day 4 and day 5. MK-2206 (1 μM) and AZD5363 (1 μM) data points only have n=2, hence no error bars. Statistics are on the relative % divided data. Student paired two-tailed t-test was performed on the data, p-values less than 0.05 are displayed.

C. Cell viability upon the CD154 + IL-21 co-culture.

Live cell viability was measured using propidium iodide (PI) intensity as described in the Methods. Student paired two-tailed t-test was performed on the data, p-values less than 0.05 are displayed.

D. Median (FSC-H) data is presented here. Average (n=3) \pm Standard deviation, days 4-5. p-values were calculated using paired two-tailed student t-test. p-values less than 0.05 are displayed.

4.10 The effect of AKT inhibitors on cell cycle regulatory molecules of CD40 + IL-21-stimulated CLL cells

AZD5363 (10 μ M) inhibited CD40+IL-4-induced proliferation of CLL cells from all three patients' samples studied. However, AKT inhibitors AZD5363 and MK-2206 inhibited CD40 + IL-21-induced proliferation of CLL cells from only 3/5 patients' samples, but not two other samples. This discrepancy obviously raised question as to why AKT inhibitors inhibit proliferation of CLL cells from some samples but not others. To answer this question, I sought to investigate the effect of AKT inhibitors on the cell cycle-related molecules involved in CD40 + IL-21-induced proliferation of CLL cells. To do this, I co-cultured primary CLL cells with CD154-expressing fibroblasts in the presence of IL-21 with or without AZD5363 (10 μ M) for 24 h, 48 h and 72 h. At the end of incubation for the indicated time periods, the co-cultured CLL cells were collected for analysis of the expression of a panel of cell cycle regulatory proteins by western blotting. CLL cells co-cultured with control fibroblasts under the similar conditions were used as a control.

First, I examined the expression of cyclins (e.g. A2, D2, D3, E1) and negative regulators of cyclin-dependent kinases (such as p27 and p21) in CLL cells before and after being co-cultured for up to three days. As expected, CLL cells from three individual samples (#3355, #3365 and #3308) all expressed hardly any cyclins A2, D2, D3 or E1 before they were cultured under co-culture conditions (Figure 4.22A-C, lane 1). Instead, these unstimulated cells all expressed significantly high levels of p27, but not p21 (Figure 4.22A-C, lane 1). These results are thus consistent with the notion that the majority of CLL cells from the peripheral blood of patients are quiescent cells, that are arrested in G₀/G₁ phase of the cell cycle (Chiorazzi et al., 2005, Zenz et al., 2010b).

Levels of p27 were reduced in CLL cells co-cultured with control fibroblasts (Figure 4.22A and B, lanes 2-4) and further reduced in CLL cells co-cultured with CD154-expressing fibroblasts + IL-21 (Figure 4.22A and B, lanes 5-7). At the same time, CLL cells co-cultured with control fibroblasts were induced to express cyclins D2 and D3, but not cyclins A2 and E1 (Figure 4.22A and B, lanes 2-4). In contrast, only the CLL cells that were co-cultured with CD154-expressing fibroblasts + IL-21 were induced to express cyclin A2 and E1 in a time-dependent manner (Figure 4.22A and B, lanes 5-7). Interestingly, addition of AZD5363 (10 μ M) inhibited induction of

cyclin A2, but not cyclin E1 (Figure 4.22A and B, lanes 8-10). Very similar results described above were also observed in repeated experiments under the same conditions but using CLL cells from the third patient sample (#3308) (Figure 4.22C). CLL sample #3308 is a re-bleed sample from the same patient whose previous sample #3106 was used in proliferation experiments and CD40 + IL-21-stimulated proliferation of CLL cells from this sample (#3106) was inhibited by 10 μ M AZD536 (Figure 4.13B and Figure 4.14A).

Quantitative analysis of pooled data obtained by densitometry thus showed that, comparing to that in unstimulated cells, the level of p27 was reduced to 40-45% in CLL cells co-cultured with control fibroblasts and further reduced to 13-20% in CLL cells co-cultured with CD54-expressing fibroblasts + IL-21 (Figure 4.22D). Addition of AZD5363 (10 μ M) restored level of p27 in CD40 + IL-21-stimulated cells, but only to 26-28% of that in unstimulated cells (Figure 4.22D). Co-culture of CLL cells with control fibroblasts induced expression of cyclins D2 and D3 and CD40 + IL-21 stimulation had little effect on the expression level of the cyclins D2 and D3 (Figure 4.22E). Addition of AZD5363 reduced but did not significantly affect the induction of cyclins D2 and D3 either (Figure 4.22E). Finally, CD40 + IL-21 stimulation selectively induced expression of cyclins A2 and E1 (Figure 4.22F). However, AZD5363 significantly reduced the expression level of cyclin A2 ($p=0.02$), but not cyclin E1 ($p>0.05$) in CD40 + IL-21-stimulated CLL cells (Figure 4.22F).

CLL cells (from case #3355) appeared to express cyclins E and A2 earlier than cells from cases #3365 and #3308 (Figure 4.22). CLL cells from case #3355 was also found to divide as early as day 5, whereas CLL cells from cases #3365 or #3106 divided on day 6-7 (Figure 4.22 and Figure 4.23). The earlier induction of cyclins E and A in #3355 may explain why it divides faster. Coincidentally, proliferation of CLL cells from case #3355 is not inhibited by AKT inhibition using either AZD5363 or MK-2206. However, whether early induction of cyclins E and A and faster cell division are responsible for the failure of AKT inhibitors to inhibit proliferation remains to be seen.

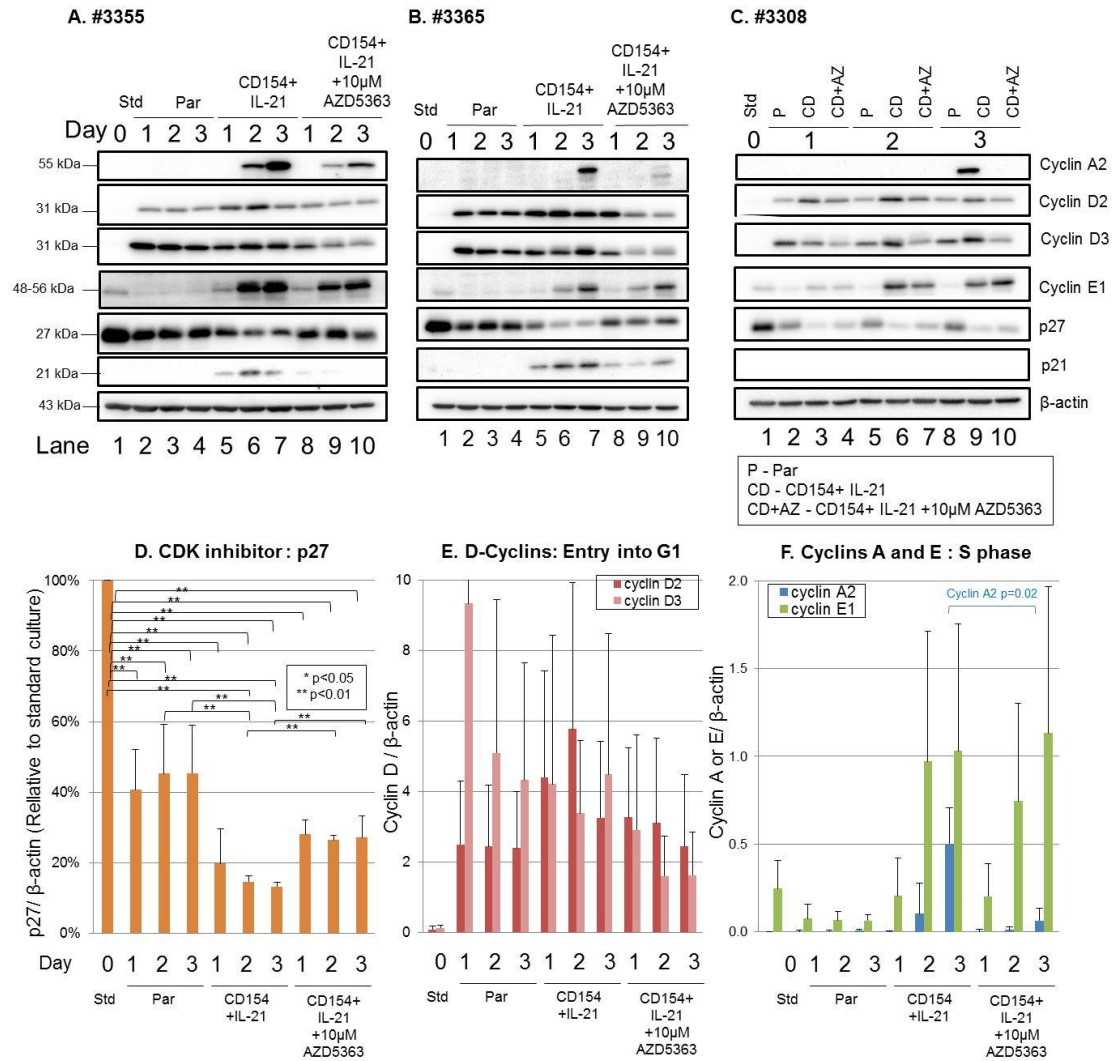


Figure 4.22: Expression of Cyclins (A2, D2, D3 and E1) and CDKis (p27, p21) in CLL cells in response to CD154 + IL-21 stimulation

- Western blot #3355.
- Western blot #3365.
- Western blot #3308.
- Average densitometry of p27 relative to standard culture / β-actin (n=3 ± standard deviation). p-values were calculated using paired two-tailed student t-test. * refers to P < 0.05, ** refers to P < 0.01.
- Average densitometry of D-cyclins: cyclin D2 and cyclin D3 absolute values / β-actin (n=3 ± standard deviation). p-values were calculated using paired two-tailed student t-test. p-values less than 0.05 are displayed.
- Average densitometry of cyclin A2 and cyclin E absolute values / β-actin (n=3 ± standard deviation). p-values were calculated using paired two-tailed student t-test. p-values less than 0.05 are displayed.

Next, I examined changes in the expression of cyclin-dependent kinases (CDK) 1, 2 and 4 in CLL cells before and after being co-cultured with control or CD154-expressing fibroblasts + IL-21. As shown in Figure 4.23A-C (lane 1), unstimulated CLL cells expressed very little amount of CDK1, CDK2 or CDK4. CDK2 was induced in CLL cells co-cultured with parental fibroblasts and CD154 + IL-21-stimulation had no effect on this induction (Figure 4.23A and B, compare lanes 5-7 to lanes 2-4). Addition of AZD5363 did not significantly affect the induction of CDK2 (Figure 4.23A and B, lanes 8-10). CDK4 was induced as early as day 1 but only in CLL cells upon CD154 + IL-21 stimulation (Figure 4.23A and B, lanes 5-7). CDK1 was also induced only in CLL cells upon CD154 + IL-21 stimulation, (Figure 4.23A, lane 7 and Figure 4.23C, lane 9). Comparing to CDK4, induction of CDK1 expression appeared to be delayed as it was only detected on day 3 after CD40 + IL-21 stimulation (Figure 4.23A, lane 7 and C, lane 9). Nevertheless, induction of CDK1 by CD40 + IL-21 stimulation was inhibited by AZD5363 (10 μ M) (Figure 4.23A lane 10 and Figure 4.23C lane 10).

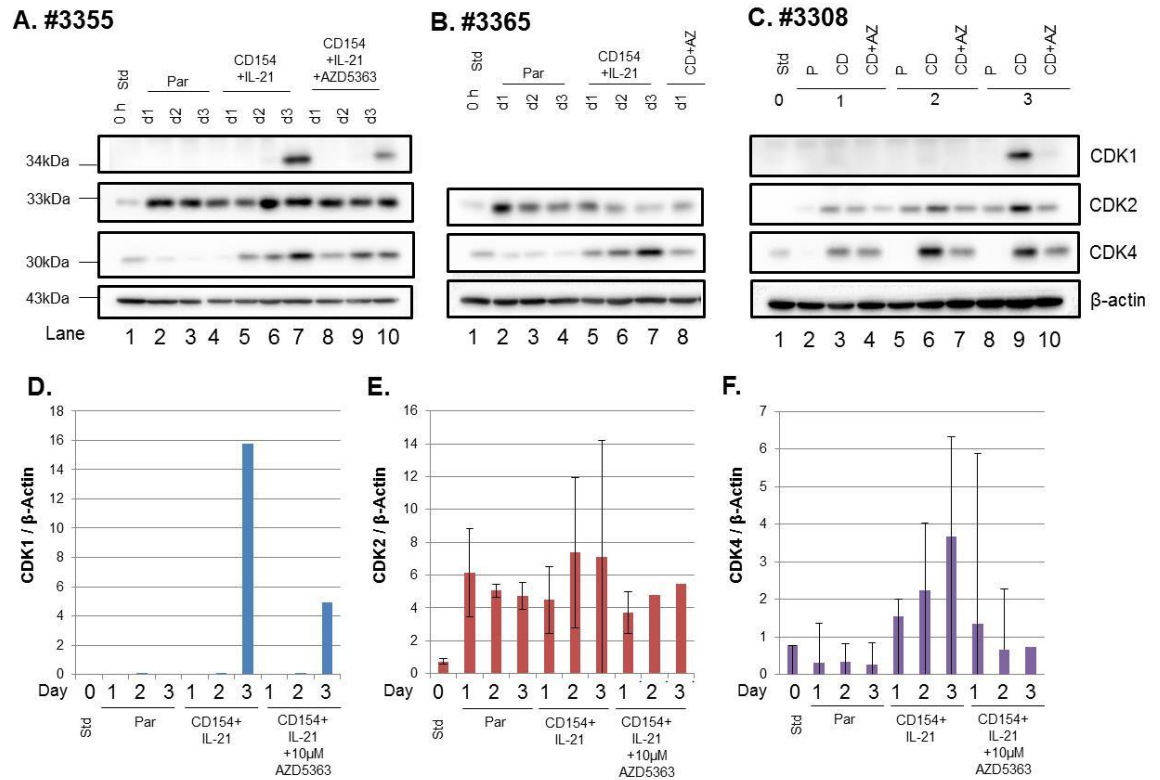


Figure 4.23: Expression of CDK1, CDK2, CDK4 in CLL cells in response to CD154 + IL-21 stimulation.

- A. Western blot #3355.
- B. Western blot #3365.
- C. Western blot #3308.
- D. CDK1 Average densitometry of absolute values / β -actin ($n=3 \pm$ standard deviation). p-values were calculated using paired 2-tailed student t-test.
- E. CDK2 Average densitometry of absolute values / β -actin ($n=3 \pm$ standard deviation). p-values were calculated using paired 2-tailed student t-test.
- F. CDK4 Average densitometry of absolute values / β -actin ($n=3 \pm$ standard deviation). p-values were calculated using paired 2-tailed student t-test.

4.11 Measuring DNA synthesis after CD154 + IL-21 stimulation

I have shown that CD40 + IL-21 stimulation selectively induced the expression of cyclin A2 and CDK1 in CLL cells and that AKT inhibitor AZD5363 inhibited induction of the two molecules (Figure 4.22). As cyclin A2 and CDK1 are required for cell cycle transition from S phase to G2 phase (completion of S phase) (Hochegger et al., 2008), together with the observation that CD40 + IL-21 stimulation-induced increase in cell size (cell growth) of CLL cells was also inhibited by AKT inhibitors (Figure 4.16), I hypothesised that inhibition of AKT will also inhibit DNA synthesis in CLL cells stimulated with CD40 + IL-21. To test this hypothesis, I used an ELISA-based assay to measure the level of BrdU incorporation into DNA of CD40 + IL-21-stimulated CLL cells in the presence or absence of AKT inhibitors. However, due to technical difficulties (mainly high background signal from the feeder layer of fibroblasts) the results obtained from the BrdU assays were inconclusive (see Appendix, 3.1 Measuring DNA synthesis after CD154 + IL-21 stimulation).

4.12 Effect of AKT shRNAs on CD154 + IL-21-induced proliferation.

In order to definitively establish that the inhibitory effect of the AKT inhibitors AZD5363 and MK-2206 on proliferation and increase in size of CD40 + IL-21-stimulated CLL cells was due to AKT inhibition, I used a lentiviral shRNA delivery technology to knockdown AKT expression in CLL cells under co-culture conditions with the help of Dr Mark Glenn in the laboratory (see detailed description of the method employing [lentiviral shRNA delivery system in Material and Methods chapter](#) and Appendix 1.5 shRNA lentiviral production).

Since there is some functional redundancy between AKT isoforms, I initially examined the role of AKT1 and AKT2 using shRNAs that specifically target AKT1 and AKT2. AKT1 has been shown to be essential for cell proliferation, while AKT2 promotes cell cycle exit. Indeed silencing AKT1 reduced cyclin A expression (Heron-Milhavet et al., 2006). A set of five clones (10162, 10163, 10174, 39794, 39797) all expressing shRNAs specific for AKT1 and five clones (563, 564, 565, 566, 39968) expressing shRNA for AKT2 were used to knockdown the two isoforms of AKT in CLL cells co-cultured with CD154-expressing fibroblasts + IL-21. A scrambled shRNA was also used as a control. As shown in Figure 4.24, clone 39794 appeared to reduce the level of AKT1 expression to 74% when compared to that

from cells treated with scrambled shRNA, and clones 564, 565 and 566 knocked down the level of AKT2 to 85%, 84% and 80%, respectively. To improve the efficiency of knockdown, we combined all the lentivirus expressing shRNAs in the hope that the pooled shRNAs would yield a greater effect in knocking down AKT 1 and/or AKT2. As shown in Figure 4.25, pooled shRNAs reduced levels of AKT1 to 90% and AKT2 to 84%, respectively. Therefore, the above results indicated that the knockdown of AKT by shRNAs was not very efficient. To determine whether this was caused by poor infection efficiency, we infected CLL cells with lentivirus that contain GFP-expressing construct and monitor the expression of GFP protein in infected cells by flow cytometry and Western blotting. As shown in Figure 4.26, GFP proteins was hardly detected in CLL cells by either method, despite high lentiviral numbers per CLL cell ($\sim 1.5 \times 10^5$ LV particles/CLL cells). This confirmed that lentiviral infection to deliver shRNA in CLL cells had not worked. I repeated these experiments twice more. Since Cantwell and colleagues had shown that the efficiency of gene transduction was enhanced five-fold by activation via CD40 cross-linking (Cantwell et al., 1996), on these occasions I stimulated the CLL cells on CD154-expressing fibroblasts for 24 hours prior to infection with approximately the same number of lentiviral particles ($\sim 1.5 \times 10^5$ LV particles/CLL cells) and on a subsequent occasion with three times the number of lentiviral particles ($\sim 4.5 \times 10^5$ LV particles/CLL cells). On both occasions there was an absence of GFP protein and no significant decrease in AKT1 or AKT2 protein expression (data not shown). As a result, I aborted the plan to knockdown AKT using lentiviral shRNA delivery approach. Ideally, these viruses should have been first tested in a cell line known to be easily infected.

#3365

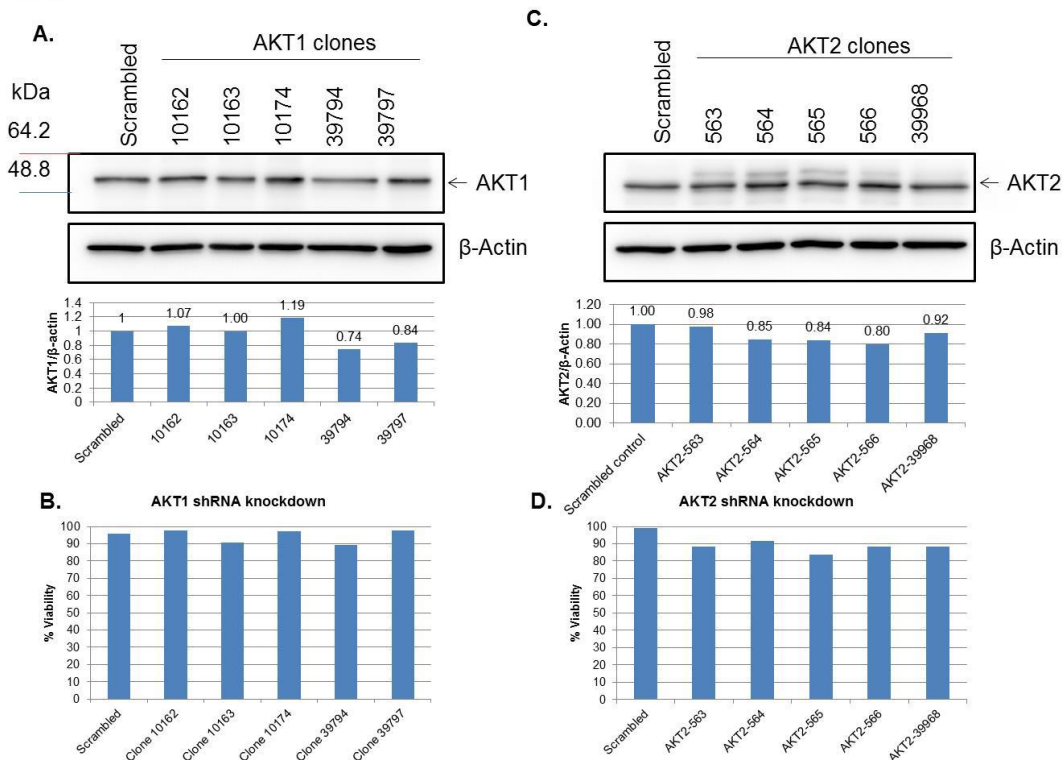


Figure 4.24: Screening individual AKT shRNA clones that can down-regulate the expression of AKT1 and AKT2.

CLL cells were plated out at 7×10^6 cells/ml in 24 well plate, virus particles were added to CLL cells and centrifuged at 1200g for 2 hours at RT. Finally, 7×10^6 CLL cells which have had virus particles added were plated out in 2ml on 6-well CD154-expressing fibroblasts. 48-72 hours post plating out, CLL cells were harvested, lysed, protein determined and western blotted for AKT1 and AKT2.

- A. AKT1 clones western blot and densitometry.
- B. AKT1 clones : % Viability data.
- C. AKT2 clones western blot and densitometry.
- D. AKT2 clones : % Viability data.

#3365

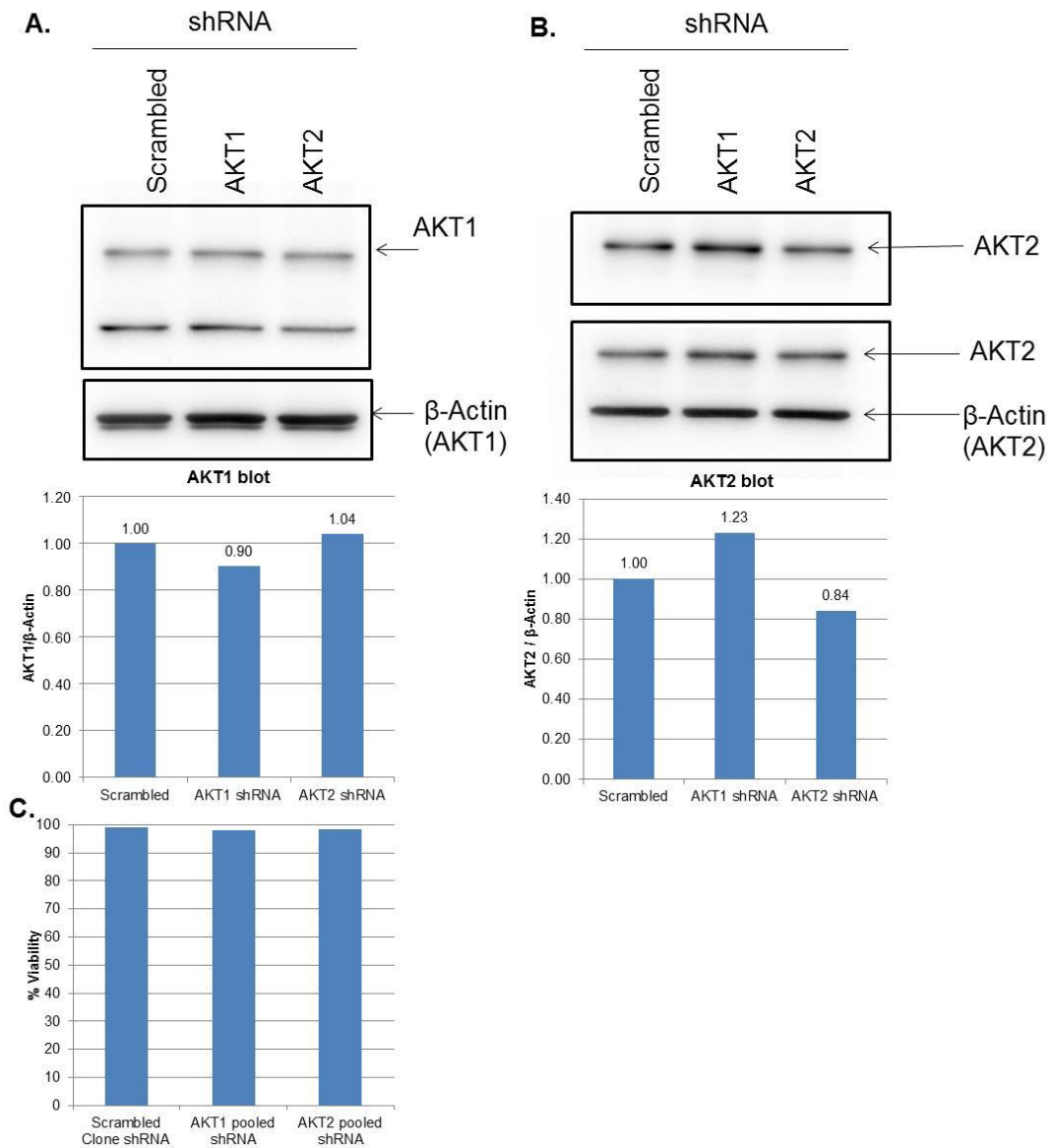


Figure 4.25: Effect of pooled AKT shRNA on the expression of AKT1 and AKT1.

Broadly, CLL cells were plated out at 7×10^6 cells/ml in 24 well plate, virus particles were added to CLL cells and centrifuged at 1200g for 2 hours at RT. Finally, 7×10^6 CLL cells which have had virus particles added were plated out in 2ml on 6-well CD154-expressing fibroblasts. 48-72 hours post plating out, CLL cells were harvested, lysed, protein determined and western blotted for AKT1 and AKT2.

- A. AKT1, western blot and densitometry.
- B. AKT2, western blot and densitometry.
- C. & % viability data.

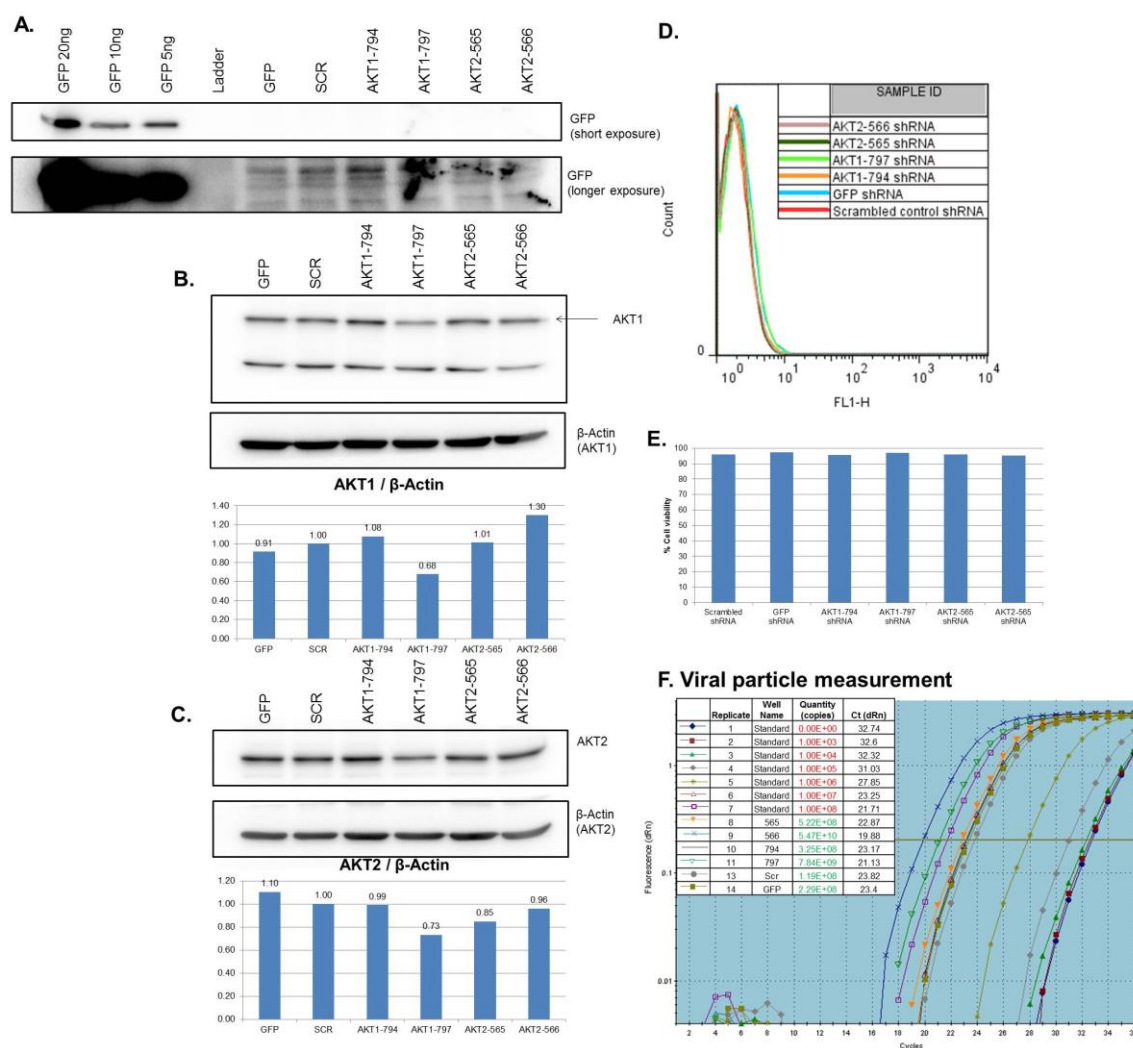


Figure 4.26: Monitoring infection efficiency of AKT shRNA in CLL cells by lentiviral delivery system

Broadly, CLL cells were plated out at 7×10^6 cells/ml in 24 well plate, virus particles were added to CLL cells and centrifuged at 1200g for 2 hours at RT. Finally, 7×10^6 CLL cells which have had virus particles added were plated out in 2ml on 6-well CD154-expressing fibroblasts. 48-72 hours post plating out, CLL cells were harvested, and GFP expression was checked using FL-1 on the flow cytometry, otherwise cells were lysed, protein determined and western blotted for AKT1 and AKT2.

- A. GFP western blot.**
- B. AKT1 western blot and densitometry.**
- C. AKT2 western blot and densitometry.**
- D. GFP Flow data.**
- E. % Cell Viability - PI Flow data.**
- F. Viral particle measurement, Ct plot from qPCR data.**

Scrambled control (lowest count) = 1.2×10^8 copies $\times 25 \times (60/10) = 1.8 \times 10^{10}$ LV particles/ μ l $\times 100 \mu$ l = 1.8×10^{12} LV particles. 1.2×10^7 CLL cells, therefore 1.5×10^5 LV particles/CLL cells.

4.13 Summary of results

I showed that AKT was required for CLL-cell growth and proliferation induced by CD40 + IL-4 as inhibition of AKT by AZD5363 significantly inhibited such cell growth and proliferation. However, unlike in CLL cells, inhibition of AKT by AZD5363 did not significantly inhibit proliferation, nor the increase in size of normal B cells in response to CD40 + IL-4 stimulation. This suggested a selective requirement for AKT in CLL-cell growth and proliferation.

I subsequently co-cultured CLL cells with CD154-expressing fibroblasts + IL-21, as the CD40 + IL-21 stimulation was reported to be a potent inducer of CLL-cell proliferation. I confirmed that CD154 + IL-21 is more potent in inducing proliferation than CD154 + IL-4. I also observed that AKT was activated by stimulation with CD40 + IL-21. In order to determine if AKT is also required for CLL-cell proliferation induced by such stimulation, I used two AKT inhibitors, namely AZD5363 and MK-2206. Both AKT inhibitors, with different modes of action, inhibited CD40 + IL-21-induced proliferation in CLL cells from three out of five of the patient samples but failed to inhibit such proliferation in CLL cells from two other patient samples. The two AKT inhibitors reduced the increase in size of CD40 + IL-21-stimulated CLL cells in all samples studied. AKT was therefore required for the CD40 + IL-21-induced growth of CLL cells in all cases studied. In contrast AKT was required for CD40 + IL-21-induced proliferation in some but not all patients' samples.

Neither AZD5363 nor MK-2206 significantly inhibited the proliferation of normal B cells induced by CD40 + IL-21 stimulation. However, the two AKT inhibitors significantly reduced the cell growth as measured by reduction in increased size of CD40 + IL-21-stimulated normal B cells.. Therefore, these results suggest that AKT is required for the growth but not proliferation of normal B cells in response to CD40 + IL-21 stimulation.

Finally, in order to understand the role of AKT in regulating cell cycle in CD40+IL-21-induced proliferation, I used Western blotting to examine the alterations in expression of the cell cycle-related molecules including some cyclins, CDKs and CDKis in CD154 + IL-21-stimulated CLL cells in the presence of the AKT inhibitor AZD5363. Consistent with literature, I showed that primary CLL cells express hardly

any cyclins or CDKs before they were stimulated with CD40+IL-21. Instead, unstimulated cells express high levels of the CDKi p27, but not p21. However, p27 expression drops rapidly whereas levels of cyclins D2, and D3, and CDK2 significantly increased in CLL cells soon after CD154 + IL-21 stimulation (day 1). Cyclins A and E, and CDK1 were also induced in the stimulated CLL cells, but their expression was detected two or three days after stimulation. Inhibition of AKT by AZD5363 significantly inhibited induction of cyclin A2 and CDK1.

4.14 Discussion

In this chapter, I showed that AKT was required for CLL-cell growth and proliferation induced by CD40 + IL-4. This is not unexpected since AKT is known to regulate growth and size via mTOR and its downstream targets S6K1 and 4EBP1/eIF4E (Fingar et al., 2002), stimulating protein synthesis and inhibiting protein degradation (Faridi et al., 2003). It is also known that AKT maintains cell size and survival by increasing mTOR-dependent nutrient uptake, which is required for protein synthesis (Edinger and Thompson, 2002). I have also shown that AKT is required for the growth, but not proliferation, of normal B cells in response to CD40 + IL-4 stimulation. This suggests that growth and proliferation are potentially two separate biological processes in normal B cells and AKT mediates the growth but not the proliferation process in normal B cells. However, the exact mechanisms underlying the selective requirement of AKT for proliferation of CLL cells in response to CD40 + IL-4 stimulation are unclear. Neither is it clear why AKT is required for the CD40 + IL-4-induced growth, but not proliferation, of normal B cells.

CLL cells are known to be smaller than normal B cells and it may be that AKT is essential for the additional growth required to enter cell cycle progression in CLL cells. CLL cells take longer than normal B cells to divide in response to CD154 + IL-4, usually taking seven to ten days rather than four to five days seen in normal B cells.

Previously, Gricks and colleagues have compared gene expression profiling of CD40 stimulated normal B and CLL cells and demonstrated a difference in dynamics of cell cycle progression in response to CD40 stimulation (Gricks et al., 2004). They showed that CDK1, cyclin B1 and cyclin F are amongst molecules that were

upregulated immediately after CD40 activation in normal B cells as compared to CLL cells. For example, following CD40 stimulation, expression of CDK1 was induced earlier in normal B cells than in CLL cells as detected by Western blotting (Gricks et al., 2004). It may be that induction of CDK1 in CLL cells is more dependent on AKT. Inhibiting AKT in CLL cells completely prevents the induction of CDK1 in CLL cells, as I have shown in CLL cells stimulated with CD40 + IL-21 (Figure 4.23), whereas perhaps in normal B cells induction of CDK1 is not so AKT-dependent. In order to fully test this hypothesis, time-course experiments to examine the expression of the CDKs in normal B and CLL cells in response to CD40 + IL-4 in the presence or absence of AKT inhibition would need to be performed.

I subsequently co-cultured CLL cells with CD154-expressing fibroblasts + IL-21, as during the course of my study a published report suggested CD40 + IL21 stimulation to be a potent inducer of CLL-cell proliferation (Pascutti et al., 2013). I confirmed that CD40 + IL-21 stimulation is indeed more potent in inducing CLL-cell proliferation than CD154 + IL-4.

CLL cells co-cultured with parental cells had reduced levels of p27 (40-45% of the level seen in unstimulated CLL cells) and expressed CDK2, cyclins D2 and D3. But they did not express cyclins A2 and E1 (Figure 4.22 and Figure 4.23). This suggests co-culture of CLL cells with parental fibroblasts can lead CLL cells to exit G0 phase and enter G1 phase of the cell cycle. However, such culture conditions are unable to induce CLL cells to complete the cell cycle as evidenced by lack of expression of cyclins A2 and E1 proteins and no reduction of fluorescence intensity in CFSE-labelled CLL cells when co-cultured with control fibroblasts.

The schematic diagram below shows a working model of cell cycle progression in CLL-cell proliferation induced by CD154 + IL-21 stimulation (Figure 4.27). CLL cells express hardly any cyclins or CDKs before they were cultured under co-culture conditions. Instead, unstimulated cells express high levels of the CDKi, p27, but not p21. Upon CD154 + IL-21 stimulation of CLL cells, levels of p27 drops rapidly on day 1 to 20%, and further down to 13% by day 2. Expression of cyclin D2 and D3, as well as CDK2 were induced from day 1. Expression of CDK4, which regulates events in early G1 phase, was induced by day 2 and its level was further increased on day 3.

Cyclin E was induced on day 2 and 3, indicating that the cells are entering S-phase around between day 2 and 3. Cyclin A2 and CDK1 both appeared on day 3, which regulates the completion of S phase, and entry into mitosis. We might then predict CDK1-cyclin B, responsible for mitosis, to be induced (though no data to support this). Cell division (proliferation) detected by reduction in CFSE fluorescence intensity was observed between day 5 and day 7 in different cases. Based on protein expression data, 10 μ M AZD5363 seems to inhibit induction of cyclin A2 and CDK1. Data on cell size showed that AZD5363 has an inhibitory effect on cell growth, therefore I believe AZD5363 is targeting AKT in G2 (but also G1) phase of the cell cycle (Figure 4.27). In mouse myoblasts silencing AKT1 reduced cyclin A expression (Heron-Milhavet et al., 2006), therefore it is not unreasonable to suggest that AZD5363 is mediating its effects through AKT1.

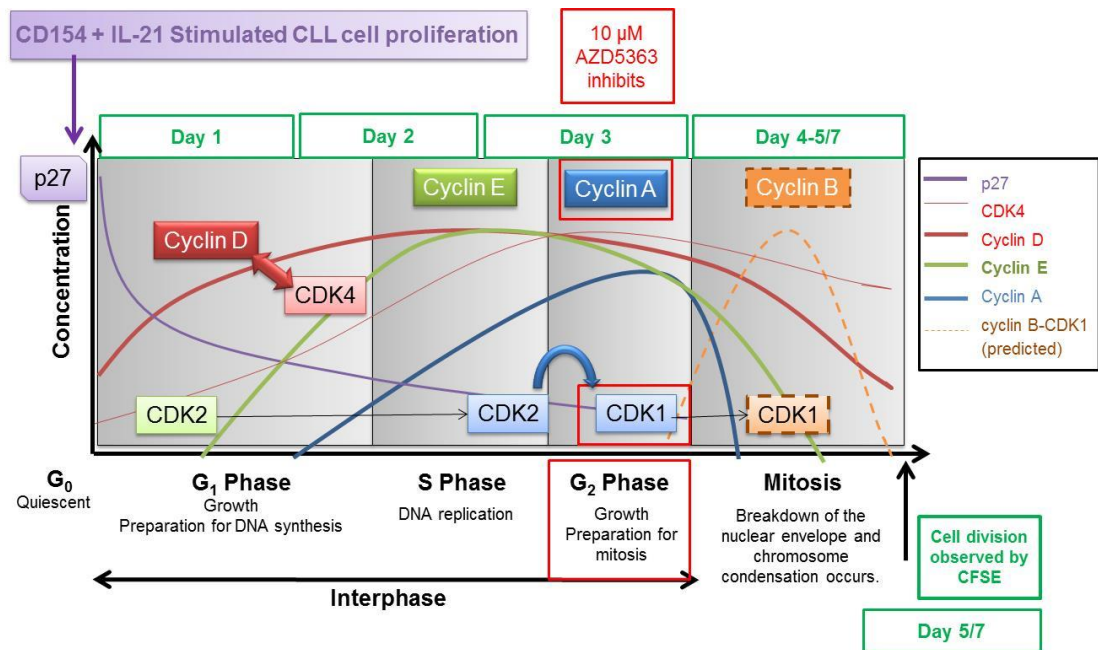


Figure 4.27: Cell cycle progression in CD154 + IL-21-induced CLL-cell proliferation

In the classical model of cell cycle, cells proceed from G₁ (growth phase), to S phase (DNA replication occurs), G₂ phase (further growth) and finally M phase or mitosis (where breakdown of the nuclear envelope and chromosome condensation occurs). The transition between these phases is controlled by cyclins and cyclin dependent kinases (CDKs). Cyclin-dependent kinases (CDKs) trigger the transitions by phosphorylating distinct sets of substrates. Cyclin D (types 1-3) and CDK4 or CDK6 regulate events in early G₁ phase, cyclin E-CDK2 triggers S phase, cyclin A-CDK2 and cyclin A-CDK1 regulate the completion of S phase, and CDK1-cyclin B is responsible for mitosis. Additional control of the entry into S phase is regulated by the CDK inhibitors; the CIP/KIP family: p21, p27 and p57 and the INK family: p16, p15 and p18. The CIP/KIP CDK family: p21, p27 and p57 inhibit Cyclin D and E, whilst INK CDKs inhibit CDK4 and CDK6 (Hocheegger et al., 2008). Upon CD154 + IL-21 stimulation of CLL cell, p27 expression drops rapidly in day 1 to 25%, and down to 10% by day 2 and 3. Cyclin D2 and D3, as well as CDK2 were expressed from day 1. CDK4, which regulates events in early G₁ phase, was induced by day 2 and increases on day 3. Cyclin E was induced on day 2 and 3, showing the cells are entering S-phase around between day 2 and 3. Cyclin A and CDK1 both appear on day 3, which regulate the completion of S phase, and entry into mitosis. We might then predict CDK1-cyclin B, responsible for mitosis, to be induced (though no data to support this). We are able to observe cell division by CFSE, between day 5 and day 7 in different cases. Based on western blot data, 10 μM AZD5363 seems to inhibit induction of cyclin A and CDK1. Cell size data shows that AZD5363 has an effect on cell growth, therefore I believe AZD5363 is mainly working in G₂ (but also G₁) phase of the cell cycle.

During the course of these experiments, another research paper was published implicating the PI3K-AKT pathway in CD40 + IL-4 induced CLL-cell proliferation. Palacios and colleagues showed that PTEN is down regulated by CD40 + IL-4 stimulation in CLL cells. In their proposed model, the proliferative behaviour of CLL cells from the subsets of progressive UM-CLL patients was due to the miR-22 expression induced after CD40 engagement. miR-22 then down regulates PTEN, leading to AKT phosphorylation (Palacios et al., 2014, Palacios et al., 2015). I found in the four CLL cases examined that AKT, as measured by phosphorylation at serine 473, was activated by stimulation with CD40 alone and level of PTEN was decreased

in CLL cells from all samples following CD40 stimulation (Figure 4.11). Addition of IL-4 or IL-21 did not appear to significantly increase extent of phosphorylation of AKT or cause further reduction of PTEN in CD40-stimulated cells. I therefore confirmed that CD40 stimulation down regulates PTEN and increases AKT activity in CLL cells.

In order to determine if AKT is also required in CLL-cell proliferation induced by CD40 + IL-21 stimulation, I used two AKT inhibitors AZD5363 and MK-2206.

With regard to the choice of concentrations of AZD5363, I had previously used 10 μ M AZD5363 in the CD40 + IL-4-induced proliferation assays and found that AZD5363 at this concentration did not induce significant cell death ([Section 3.2.2](#), Figure 3.3B). Since the IC₅₀ for inhibiting 50% GSK phosphorylation was 3 μ M AZD5363 ([Section 3.2.2](#) Figure 3.3), I wanted to see if lower doses of AZD5363 (1-3 μ M) might also inhibit CD40 + IL-21-stimulated proliferation. Therefore I chose to use 1, 3 and 10 μ M AZD5363.

To confirm that the effects observed with AZD5363 were specific to AKT inhibition, I used another AKT inhibitor MK-2206, which inhibits AKT activation by binding to the PH domain of the enzyme, thus preventing its translocation to the membrane (Hirai et al., 2010). MK-2206 had been shown to induce apoptosis of CLL cells *in vitro*, with 8 μ M concentration of the drug being able to kill 50% of primary CLL cells after 72 hours of incubation (Ding et al., 2013). I therefore used 1, 3 and 10 μ M MK-2206 in my experiments.

Both MK-2206 and AZD5363 inhibited CD154 + IL-21 induced proliferation in CLL cells from three of the five cases as measured on day 7 (#3091, #3106, #3365; Figure 4.13A-C,F-H; Figure 4.14A&D). This suggests that these three cases of CLL cells are particularly dependent upon AKT for proliferation and there may be therapeutic potential for AKT inhibition in these cases of CLL.

However, such proliferation was not inhibited by the two AKT inhibitors in CLL cells from two other patient samples (#2814 and #3355). CLL cells from case #2814 expressed unmutated *IGHV* (0%) whilst those from case #3355 expressed mutated *IGHV* (3.4% mutated). This suggests that the ability of AZD5363 to inhibit proliferation does not correlate with *IGHV* mutational status of CLL cells. However,

CLL cells from these two cases, #3355 (Figure 4.13D and I) and #2814 (Figure 4.13E and J), all began to proliferate as early as day 5 in response to CD40 + IL-21 stimulation.

AZD5363, if anything, caused an increase in proliferation of CLL cells from cases #3355 (Figure 4.13D and I) and #2814 (Figure 4.13E and J) upon CD154 + IL-21 stimulation. AZD5363 (at 1, 3, 10 μ M) caused an increase in proliferation in #2814 and #3355 (Figure 4.14B-C). MK-2206 at 1 μ M concentration also caused an increase in proliferation of CLL cells from cases #2814 and #3355, but at 3 μ M MK-2206 did not have such effect (Figure 4.14E-F). CLL cells from these two cases also seemed to be the most sensitive to the highest dose of MK-2206 (10 μ M) which caused significant cell death as measured by PI/flow cytometry method, which resulted in decreased proliferation (Figure 4.14E-F).

It is worth noting that in CLL cells from case #2814, where proliferation was inhibited by AZD5363 in the CD154 +IL-4 proliferation assay (Figure 4.2A&B), proliferation was similarly not inhibited by either AZD5363 or MK-2206 in the CD154 + IL-21 proliferation assay (Figure 4.13E and J). This implies that CD154 + IL-21-induced proliferation was less dependent upon AKT, at least in this sample.

The mechanisms of why CLL cells from some cases, but not others, require AKT for CD40 + IL-21-induced proliferation are currently unknown, but most likely involve multiple factors. Nevertheless, I would suggest the following five avenues to explore: (a) increasing the sample size; (b) detecting mutations in genes commonly identified in CLL by next generation sequencing of DNA; (c) gene expression profiling of CLL cells upon CD40-stimulation with and without AKT inhibitors; (d) examining the levels of surface expression of CD154, CD40 and IL-21R in these CLL cells; and (e) examining levels of TCL1 and other proteins that are complexed with AKT at the plasma membrane.

Firstly, I would increase the sample size from five samples to a larger number as increasing the cohort size of samples with well annotated clinical features may bring out clearer trends. In addition, in recent years many additional genetic mutations in genes such as *ATM*, *NOTCH1*, *SF3B1* and *BIRC3* as well as *TP53* have been discovered that affect the clinical outcome of CLL (see introduction section (1.1.3.4 Recurrent mutations in CLL). These mutations also likely affect signalling within the

CLL cells and detecting these mutation by next generation sequencing may potentially explain why inhibition of AKT inhibits proliferation in some cases, but not others. BFL-1, BCL-XL and BID were normally upregulated in SF3B1 and ATM mutated CLL cells, indicating that at least the CD40-mediated NF- κ B response is not affected by these mutations (Te Raa et al., 2015). However whether the transcription of genes is altered post CD40 + IL-4 or IL-21 in these mutated cases has not been investigated.

Gene expression profiling of CLL cells upon CD40-stimulation with and without AKT inhibitors with stimulated CLL cells collected at times earlier than the time of division may yield important information regarding the differences in expression of genes between the CLL cases. It is known that CLL cells do have a differential response to soluble CD154 (sCD154) stimulation. sCD154 leads to the upregulation of CD95 and CD80 and chemokines CCL22 and CCL17 in some, but not all cases (Scielzo et al., 2011). In the cases where CLL cells were induced to produce chemokines and upregulate surface markers, signalling was occurring via NF κ B pathway resulting in BCL-2 and MCL1 expression (Scielzo et al., 2011).

Examining the levels of surface expression of CD154, CD40 and IL-21R in these CLL cells may explain why #2814 and #3355 seem to proliferate faster as, if they express CD154, these cells would already have autocrine or paracrine signalling in operation. CLL cells express low levels of CD154 in one third of cases (Schattner, 2000). It may be that the cases which are not inhibited by AKT inhibitors have already been experiencing autocrine or paracrine CD40 signalling. CD40 stimulation increases IL-21R expression in CLL cells (de Toter et al., 2006) and CLL cells from #2814 and #3355 which seem to proliferate faster may also potentially express more IL-21R.

Examining levels of TCL1 or other proteins that are complexed with AKT at the plasma membrane might be another avenue to pursue. It may be that high TCL1 levels attenuate the effect of AKT inhibition in CLL cells from cases such as #2814 and #3355. AKT directly interacts with TCL1 and is critical for AKT activation in CLL cells (Hofbauer et al., 2010). High levels of TCL1 are indicative of high BCR responsiveness and adverse outcome (Herling et al., 2009). Disrupting AKT- TCL1 interaction may have therapeutic potential (Popal et al., 2010). In fact, peptide-based

TCL1-interphase mimics were potent in steric AKT antagonization and in reducing CLL cell survival (Schrader et al., 2014). In addition, examining additional AKT-binding proteins by, for example, co-immunoprecipitation and Western blotting might be another approach to examining other proteins that may be complexed with AKT.

Pascutti and colleagues showed that the addition of IL-21 to the CD154-fibroblast co-culture led to STAT3 phosphorylation and thus implicated the JAK-STAT pathway as a possible target for therapeutic intervention. Pascutti and colleagues also showed that a JAK inhibitor almost totally inhibited CD154 + IL-21 induced proliferation (Pascutti et al., 2013). Compared to the almost total inhibition of proliferation observed when a JAK inhibitor was used, the effect of inhibition of AKT was modest, even in the three cases where proliferation was inhibited. In the three cases where CD154 + IL-21 induced proliferation was inhibited, 10 μ M AZD5363 and 3 μ M MK-2206 reduced proliferation to 30% on day 5, rising to 40% of day 6 and finally 50% on day 7 (Figure 4.14A&D), compared to the reported almost total inhibition (from 40% to 5%) with the JAK inhibitor, Ruxolitinib after five days (Pascutti et al., 2013). Therefore, perhaps AKT is not as important as the JAK-STAT pathway in mediating the CD154 + IL-21 signalling that leads to proliferation.

AKT inhibition by the two inhibitors inhibited the increase in size of CD40 + IL-21-stimulated CLL cells in all samples studied. AKT is therefore required for CD154 + IL-21-induced growth of CLL cells. This clearly shows that whilst cell growth is generally considered essential for cell division, these two processes are separable, since CLL cells from the two cases (#2814 and #3355) whose cell division was not inhibited by AKT inhibitors displayed decreased cell growth with the addition of AKT inhibitors. However, exactly how these two processes are co-ordinated is still incompletely understood (Fingar et al., 2002).

Neither AZD5363 nor MK-2206 inhibited the proliferation of normal B cells induced by CD40 + IL-21 stimulation. However, the two AKT inhibitors significantly reduced the growth as measured by increase in size of CD40 + IL-21-stimulated normal B cells. Therefore, these results suggest a distinct role of AKT in cell growth and proliferation of normal B cells in response to CD40 + IL-21 stimulation.

Finally, I examined the expression of the cell cycle regulatory molecules (cyclins, CDKs and CDKis) in CD40 + IL-21 stimulated CLL cells in order to understand underlying mechanisms mediating CD40 + IL-21-induced CLL-cell proliferation and the effect of AKT inhibition by AZD5363. I first sought to determine if the anti-proliferative effect of AZD5363 might be via the induction of p21. Lenalidomide was shown to inhibit CLL-cell proliferation induced by CD154 + IL-4 + IL-10 in a cereblon/p21-dependent manner (independent of p53) (Fecteau et al., 2014). Although the precise mechanism mediating p21 upregulation by lenalidomide is unclear, increased p21 expression ultimately leads to cell-cycle arrest of CLL cells (Fecteau et al., 2014, Kater et al., 2014).

p21 is known to be a downstream substrate of AKT through both direct and indirect phosphorylation. When it is activated, AKT can directly phosphorylate p21 at T145 and S14b, facilitating the export of p21 out of nucleus into cytoplasm where it is ubiquitinated and degraded. This allows CDKs to restore their kinase function in cell cycle progression and help the cells proceed through G1 phase. AKT also inhibits p21 expression indirectly through its phosphorylation and activation of MDM2 and subsequent down-regulation of p53-mediated transcription of p21 (Mayo and Donner, 2001, Zhou et al., 2001b).

Therefore one would expect that addition of an AKT inhibitor would result in the increased expression of CDKis (such as p21, or p27), either directly or indirectly (via p53) resulting in cell cycle inhibition. However, I was unable to detect p21 in resting CLL cells and could only detect it at low levels in CD154 + IL-21-stimulated CLL cells. Addition of AZD5363 restored the expression of p27, but had no effect on the expression of p21 (Figure 4.22A&B).

Chapter 5 : Regulation of AKT activity by PI3K in CLL

5.1 Background and aims

Having explored the function of AKT in CLL-cell survival and proliferation, I next studied its activation in CD40-stimulated CLL cells. AKT is known to be activated by many cytokines and chemokines present in the lymph node and bone marrow microenvironment ([Section 1.4](#)). I previously showed that phosphorylation of AKT in CLL cells is maintained upon CD40-stimulation ([Section 3.3](#)).

As AKT is generally considered to be a key downstream effector kinase of PI3K, as described in Chapter 1 ([Section 1.3.2.1](#)), the aims of this part of my PhD study were to determine whether the activation of AKT by microenvironment factors such as CD40 stimulation was mediated by PI3K, and if so, which isoform(s) is(are) responsible.

To address these questions, I first examined the expression of class I PI3K p110 isoforms in primary CLL cells in comparison with normal B cells. I then examined changes in the expression of the class I PI3K p110 isoforms between CLL cells co-cultured with CD154-expressing fibroblasts versus control fibroblasts. Finally, I used pharmacological inhibitors selective to p110 isoforms of the class I PI3Ks to determine which isoform(s) was responsible for activating AKT in response to CD40 stimulation.

5.2 The expression of isoforms of class I PI3Ks in CLL cells in comparison with normal B cells

The class I PI3Ks are composed of two subunits: the catalytic subunit (p110 α , β , δ , γ) and the regulatory subunit (p85 α and p85 β ; p55 α , p50 α , p55 γ or, in the case of the catalytic subunit p110 γ , p101 or p87) (see [section 1.2](#)). I focused on protein expression of catalytic p110 subunits as they most closely relate to the activity of the enzyme (Vanhaesebroeck et al., 2012).

5.2.1 Optimisation of detecting class I PI3K p110 isoforms in CLL cells by Western blotting

In order to determine which isoforms of the class I PI3Ks are present in CLL cells, I used Western blotting to detect protein expression using commercially available

antibodies against the relevant isoforms, and recombinant human proteins p110 α , β , δ and γ (provided by AstraZeneca) as positive controls. Cellular proteins in lysates prepared from primary CLL cells were separated on a 10% SDS-PAGE gel alongside each recombinant protein and transferred to PVDF membranes, which were first probed for p110 α and p110 δ , individually, and then stripped and re-probed for p110 β , and p110 γ , respectively. β -Actin was probed as a loading control.

As shown in Figure 5.1A, the primary antibodies appeared to be specific as they detected the respective p110 recombinant proteins on the membranes with all bands displaying the expected molecular weights, (i.e. slightly below the 115 kDa marker of the pre-stained protein ladders) except for the p110 γ antibody (see explanation below).

My initial results also showed that class I PI3K p110 α isoform was abundantly expressed in CLL cells whereas p110 β isoform was expressed at a lower level (Figure 5.1A). As expected, p110 δ isoform was also detected in CLL cells.

Interestingly, the p110 δ antibody detected two bands in the CLL samples, one with the expected molecular weight of 110 kDa, and the other with a molecular size of ~ 37 kDa. It was initially considered that the product with lower molecular weight might be the reported p110 δ splicing isoform p37 δ (Fransson et al., 2012). Further examination, however, suggested that this possibility is very unlikely. The p110 δ antibody from Abcam was raised against an immunogen consisting of 20 amino acids at the C-terminal region of PI3K p110 δ , whereas the reported splicing variant p37 δ protein contains the p85-binding domain at the N-terminal and a truncated RAS-binding domain, but lacks the catalytic domain at the C-terminal region of the full length p110 δ (Fransson et al., 2012). Consequently, the p110 δ antibody from Abcam should not detect the splicing product p37 δ . Therefore, there are several possible explanations for the appearance of this product of lower molecular size: it could be a cleavage product of p110 δ within the cells, or a degraded product as a result of sonication and/or boiling of the protein samples for SDS-PAGE.

Alternatively, it could simply be a non-specific band.

The p110 γ antibody did not detect recombinant p110 γ protein, which was used as a positive control, but did detect a protein with a molecular weight of about 110 kDa in all three CLL samples (Figure 5.1A). To test the possibility that the failure to detect

signals in positive control samples was caused by the degradation of the recombinant protein, I repeated the Western blot by loading 16.4 μ g recombinant p110 γ protein (i.e. 328-fold more than 50 ng initially used) and observed that the recombinant protein was indeed mostly degraded with the intact full length p110 protein hardly detected on the blot, but many resultant degraded products of smaller molecular weights (Figure 5.1B).

As the p110 γ antibody did not cross-react with any other isoforms of the class I PI3Ks (Figure 5.1A), I felt confident that the antibody was specific to p110 γ and continued using this antibody for future experiments.

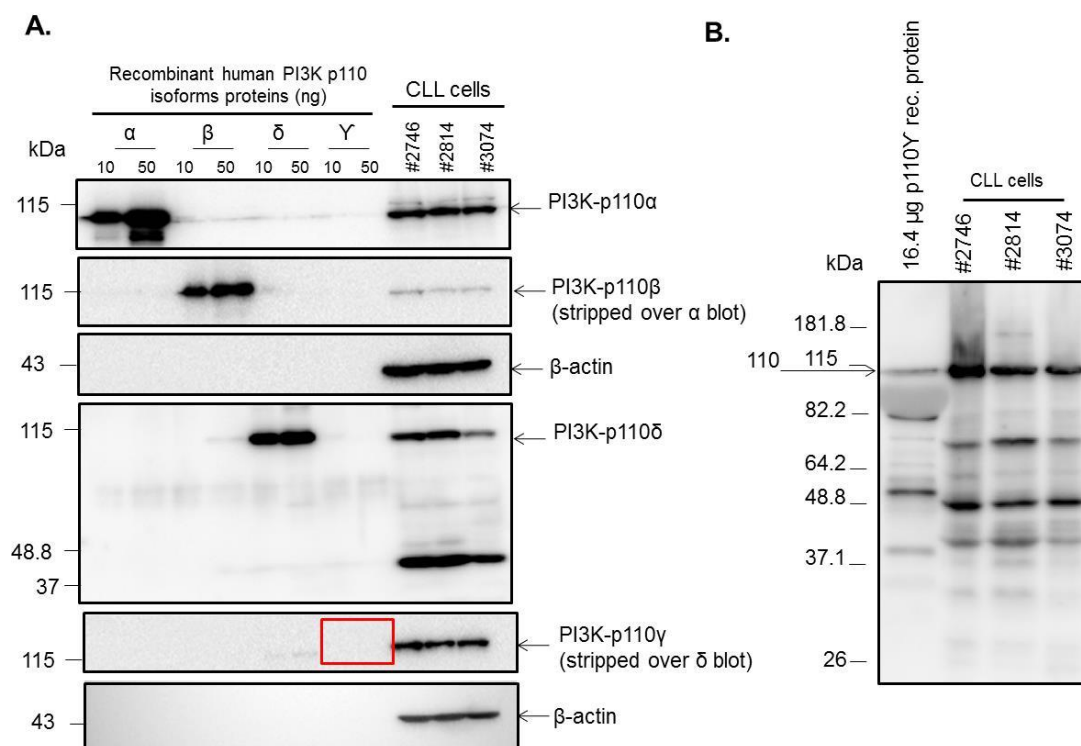


Figure 5.1: Positive controls for p110 PI3K isoforms.

A. Positive controls for p110 PI3K isoforms.

CLL cells from three cases were thawed and recovered for 1 hour. 4×10^6 Cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in 100 μ l of break-up buffer (10mM Tris-HCl, pH7.4, 5mM MgCl₂, 1% Triton X-100, 0.1M NaCl), and was pulse sonicated manually. 25 μ l of cells were loaded along with 10 and 50 ng of each recombinant protein: p110 α , β , δ and γ , onto two 10% SDS-PAGE gels. The SDS-PAGE gel was run and proteins were transferred onto PVDF membrane. The membranes were incubated in primary antibodies overnight, PI3K p110 α (C73F8) and PI3K p110 δ Abcam (ab1678); followed by relevant HRP-conjugated secondary antibodies and the Western was developed using ECL on a CCD camera. Blots were stripped in buffer containing 62.5 mM Tris-HCl (pH 6.7), 2% SDS and 100 mM 2-mercaptoethanol at 50°C for 30 min. Membranes were re-probed with PI3K p110 β (C33D4) and PI3K p110 γ (D55D5) CST. β -Actin served as a loading control.

B. p110 γ recombinant protein is degraded.

CLL cells from three cases were thawed and recovered for 1 hour. 4×10^6 Cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in 100 μ l of break-up buffer (10mM Tris-HCl, pH7.4, 5mM MgCl₂, 1% Triton X-100, 0.1M NaCl), and was pulse sonicated manually. 25 μ l of cells were loaded onto a gel, alongside 16.4 μ g of p110 γ recombinant protein (more than 328-fold more than 50 ng) obtained from AstraZeneca. The SDS-PAGE gel was run and proteins transferred onto PVDF membrane. The membrane was incubated in PI3K p110 γ (D55D5) antibody overnight; followed by relevant HRP-conjugated secondary antibodies and the Western was developed using ECL on a CCD camera.

5.2.2 The expression of class I PI3K p110 isoforms in CLL cells

Having optimised the conditions of detecting the class I PI3K p110 isoforms by Western blotting, I set out to examine the expression of these isoforms in CLL cells obtained from more cases. For comparison, I also included normal B cells isolated from the PBMCs of healthy donors using a negative selection method (MACS B Cell Isolation Kit II, catalogue no. 130–091–151, from Miltenyi Biotec Ltd). The purity of CD19⁺ B cells was determined to be greater than 90.0% (see Figure 2.3 and Table 2.3 in Methods and Materials chapter). Therefore, CLL cells from ten different patient samples were used, together with three normal B cell samples. Cells were lysed in lysis buffer and equal volume of cellular lysates loaded onto four separate gels for the detection of each p110 isoform. β -Actin was probed as a loading control. Densitometry was performed for quantification of the signals of interest and Mann-Whitney U tests were performed to determine the statistical significance of the difference in the data from the two groups.

As shown in Figure 5.2A, PI3K p110 α was expressed a similar level across the samples of CLL cells and normal B cells. Statistical analysis showed that there was no significant difference in the expression of p110 α between the two cell types ($p=0.1608$). In contrast, PI3K p110 β was expressed at a lower level in CLL cells as compared to normal B cells and this difference was statistically significant ($p=0.007$) (Figure 5.2B). Meanwhile, PI3K p110 δ appeared to be more abundantly expressed in CLL cells than in normal B cells and this increase was statistically significant ($p=0.007$) (Figure 5.2C). Finally, the expression in PI3K p110 γ appeared to be significantly higher in CLL cells as compared to the negligible amount seen in normal B cells and again this increase was statistically significant ($p=0.007$) (Figure 5.2D).

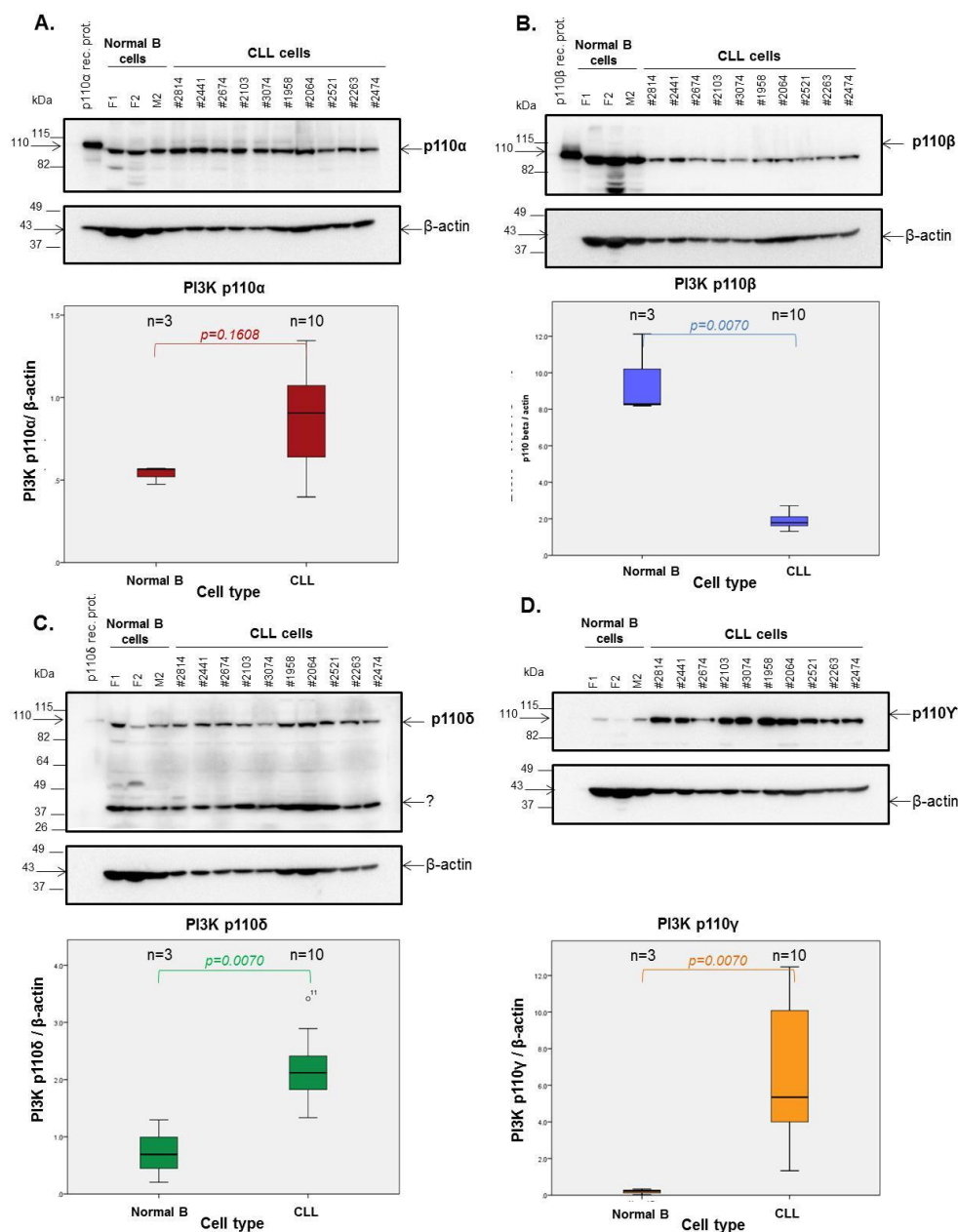


Figure 5.2: p110 isoforms expression in normal B and CLL cells.

Normal B cells from healthy donors were isolated using a negative selection method, the MACS B Cell Isolation Kit II (Cat. No. 130-091-151, Miltenyi Biotec ltd), and the CD19+ purity was determined to be greater than 90.0%. CLL PBMC cells from ten cases were thawed and recovered for 1 hour. 4×10^6 normal B or CLL PBMC cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in 100 μ l of break-up buffer (10mM Tris-HCl, pH7.4, 5mM MgCl₂, 1% Triton X-100, 0.1M NaCl), and was pulse sonicated manually. 25 μ l of cells along with 10 ng of recombinant protein were loaded onto four 10% SDS-PAGE gels. The SDS-PAGE gels were transferred onto PVDF membrane. The membranes were incubated in primary antibodies overnight, and followed by relevant HRP-conjugated secondary antibodies and the Western was developed using ECL on a CCD camera. β -Actin served as a loading control. Densitometry was performed using AIDA image analyser. IBM® SPSS® Statistics Version 21, Release 21.0.0.1. 64-bit edition was used to create box-plots and perform Mann-Whitney U tests. p-values displayed.

- A. PI3K p110α.
- B. PI3K p110β.
- C. PI3K p110δ.
- D. PI3K p110γ.

5.2.3 CLL cells express class I PI3K p110 γ

The expression of PI3K p110 γ isoform in normal B cells has not been well studied. This may stem from an early report from animal studies that PI3K p110 γ plays no role in the development or function of B cells (Sasaki et al., 2000). At the mRNA level, PI3K p110 γ expressed by normal B cells was found to be at a significantly lower level than that in dendritic cells, natural killer (NK) cells and myeloid cells, and below the average expression from a panel of 79 human tissues (Su et al., 2004). To my knowledge, expression of PI3K p110 γ at protein level in normal B cells has not been reported. Similarly, I was not aware of any report on the expression of PI3K p110 γ protein in CLL cells at the time. To ensure that the PI3K p110 γ detected in the CLL samples was not a result of contamination by dendritic, NK and myeloid cells, I purified the CLL cells using a negative selection method (MACS B-CLL Cell Isolation Kit, catalogue no. 130–103–466, from Miltenyi Biotec Ltd). The purity of B-CLL cells was assessed by percentage of cells that were double positive for CD5 and CD19 on FACS (Table 2.2 and Figure 2.2 in Methods and Material chapter). For comparison, I also included normal B cells which were isolated using the negative selection method from PBMCs prepared from the Buffy Coats (purchased from NHS Blood and Transplant Speke, Merseyside) and were shown to be greater than 93% CD19 positive (Table 2.4 and Figure 2.4). Three such isolated normal B cell samples and five pre- and post-purification CLL cell samples were examined using Western blotting for the expression of PI3K p110 γ (Figure 5.3). As shown in Figure 5.3, CLL cells from four patient samples (#3091, #3355, #3363 and #3365) contained greater than 97% CD5 & CD19 double positive cells post purification. In contrast, CLL cells from sample #3353 showed only 63.4% of the population was double positive for CD5 & CD19 post purification even though 99.9% of these cells were positive for CD19 (Table 2.2 and Figure 2.2 in Methods and Material chapter). This CLL sample expressed surface immunoglobulin G type instead of M type seen in most CLL samples (Figure 2.2 in Methods and Materials chapter), indicative of cells undergoing immunoglobulin class switch. Nevertheless, PI3K γ was clearly detected in both pre- and post-purified CLL cells (Figure 5.3). Again, the level of PI3K γ was higher in CLL cells than normal B cells (although this time not

statistically significantly). Therefore, these results showed that PI3K p110 γ was expressed by CLL cells.

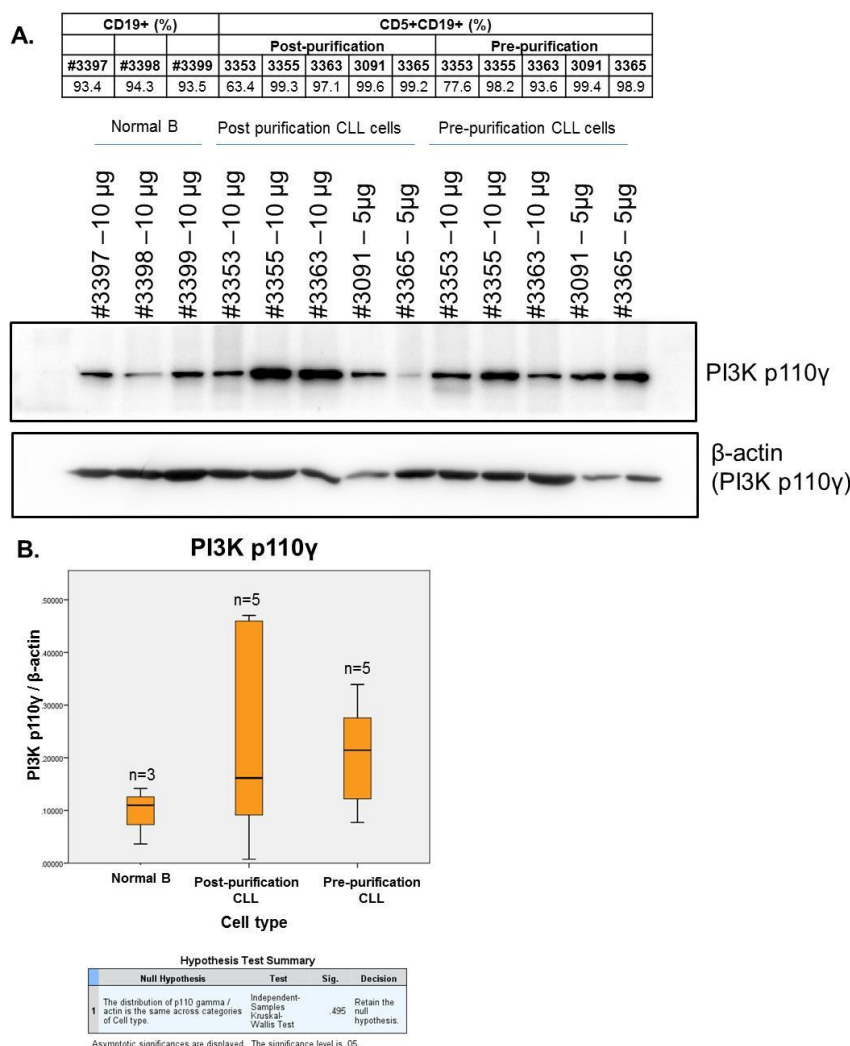


Figure 5.3: PI3K p110 γ was present in purified CLL cells.

Normal B cells were isolated using a negative selection method (MACS B Cell Isolation Kit II, # 130–091–151, Miltenyi Biotec Ltd) from PBMCs prepared from Buffy Coats purchased from NHS Blood and Transplant, and were shown to contain greater than 93% CD19 $^{+}$ cells. CLL cells were purified using the negative selection human B-CLL Cell Isolation Kit (130-103-466, Miltenyi Biotec Ltd). B-CLL Cell CD5+CD19 $^{+}$ expression was assessed as a marker of purity. Three normal B cells (from buffy coats) and five pre- and post- purification CLL cells were examined via Western blotting for their PI3K p110 δ and PI3K p110 γ expression. 4 x 10 6 normal B or CLL PBMC cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in 100 μ l of RIPA buffer and sonicated using a water sonication device. Protein concentration of the lysates was determined using Bradford assay and 5 μ g or 10 μ g of cellular proteins were loaded onto a 10% poly-acrylamide gel and separated by SDS-PAGE (as labelled). The separated proteins on the gel were transferred onto PVDF membrane. The membranes were incubated in primary antibodies overnight followed by relevant HRP-conjugated secondary antibodies, and the Western was developed using ECL on a CCD camera. β -Actin served as a loading control. Densitometry was performed using AIDA image analyser. IBM® SPSS® Statistics Version 21, Release 21.0.0.1. 64-bit edition was used to create box-plots and perform Kruskal-Wallis tests.

A. Western blot.

B. Box-plots of p110 γ expression and Kruskal-Wallis test showing non-significant.

5.3 The effect of CD40 stimulation on expression of class I PI3K p110 isoforms in CLL cells

Having shown that primary CLL cells expressed all four class I PI3K p110 isoforms, I next asked the question of whether PI3K p110 isoform expression was altered by CD40-stimulation. To this end, I co-cultured primary CLL cells with parental control or CD154-expressing fibroblasts for 24, 48 and 72 hours. At the end of incubation, CLL cells were harvested, lysed and 10 µg of cellular proteins loaded onto 10% poly-acrylamide gel and subjected to SDS-PAGE. Proteins were then transferred onto PVDF membranes which were probed for the p110 isoforms. BCL-XL was probed as a marker for CD154 stimulation.

As shown in Figure 5.4, PI3K p110 α isoform was detected in all CLL cells cultured under standard condition or on co-cultures. Compared to CLL cells co-cultured with parental fibroblasts, CLL cells co-cultured with CD154-expressing fibroblasts expressed decreased amount of PI3K p110 α 24 hours after co-culture (78% compared to 100% in parental control). By 48 and 72 hours, the expression of p110 α in CD40-stimulated CLL cells was significantly reduced (to about 50% or less of the levels in CLL cells co-cultured with parental fibroblasts) with p values of p=0.02 and p=0.03, respectively (Figure 5.4)

The expression of PI3K p110 β was detected in all unstimulated CLL samples and its expression did not appear to be significantly altered by CD40 stimulation (Figure 5.5). In contrast, PI3K p110 δ isoform can be readily detected in unstimulated CLL cells and its expression was slightly increased in CLL cells cultured under standard conditions for 24 h (Figure 5.6). Comparing to cells co-cultured with parental fibroblasts, CLL cells co-cultured with CD154-expressing fibroblasts for 24 h expressed higher level of PI3K p110 δ isoform (i.e. 1.54-fold increase, even though this was not statistically significant with a p-value of 0.09) (Figure 5.6). However, this increase appeared to disappear over time and by 72 h there was no difference in the expression of PI3K p110 δ isoform in CLL cells co-cultured with either parental or CD154-expressing fibroblasts.

As expected, PI3K p110 γ isoform was also readily detected in unstimulated CLL cells and its expression was not affected by incubation over 24 h under standard culture conditions (Figure 5.7). However, similar to PI3K p110 δ , the expression of

PI3K p110 γ isoform in CLL cells co-cultured with CD154-expressing fibroblasts for 24 h increased by 1.53-fold as compared to that in CLL cells co-cultured with parental fibroblasts (Figure 5.7). This increase at 24 h time point was statistically significant ($p=0.04$) (Figure 5.7). However, the increase in the expression of PI3K p110 γ disappeared over time and, by 48 and 72 h, there was no significant difference in the expression of PI3K p110 γ in CLL cells with or without CD40 stimulation.

Taken together, the above results showed that CD40 stimulation decreased expression of PI3K p110 α whilst having no effect on the expression of PI3K p110 β in CLL cells. In contrast, the expression of both PI3K p110 δ and p110 γ isoforms was up-regulated in CD40-stimulated CLL cells. Increased expression of both isoforms occurred early in response to CD40 stimulation and this increase lasted less than 48 h.

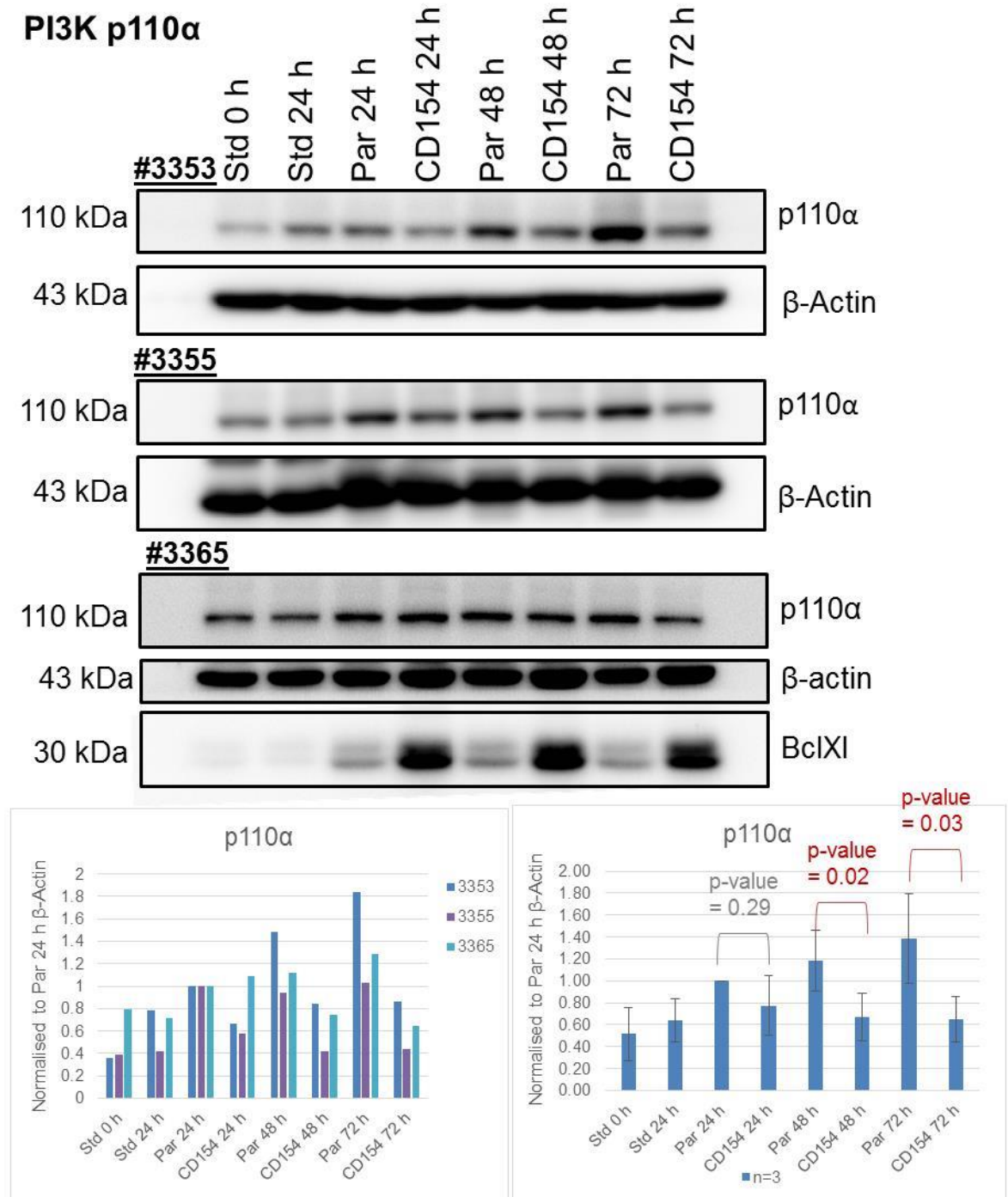


Figure 5.4: p110α isoform expression upon CD40-stimulation of CLL cells.

CLL cells from three cases were thawed and recovered for 1 hour (Standard culture 0 h, Std 0 h), and cultured for 24 hours either alone (Standard culture 24 h, Std 24 h), co-cultured on parental fibroblasts (Par), or co-cultured with CD154-expressing fibroblasts (CD154) for 24 hours (24h), 48 hours (48h) or 72 hours (72h). Cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in modified RIPA with sonication, protein determination performed and 10 µg of cell lysate loaded onto 10% SDS-PAGE gel. Proteins were transferred onto PVDF membrane. The membrane was incubated in primary antibodies overnight, followed by relevant HRP-conjugated secondary antibodies and the Western was developed using ECL on a CCD camera. Densitometry was performed using AIDA image analyser. β-Actin served as a loading control. Bcl-XL served as a positive control for CD154 stimulation. Graph shows the mean ± SD. p-values were calculated using the student two-tailed paired t-test; p-values are displayed.

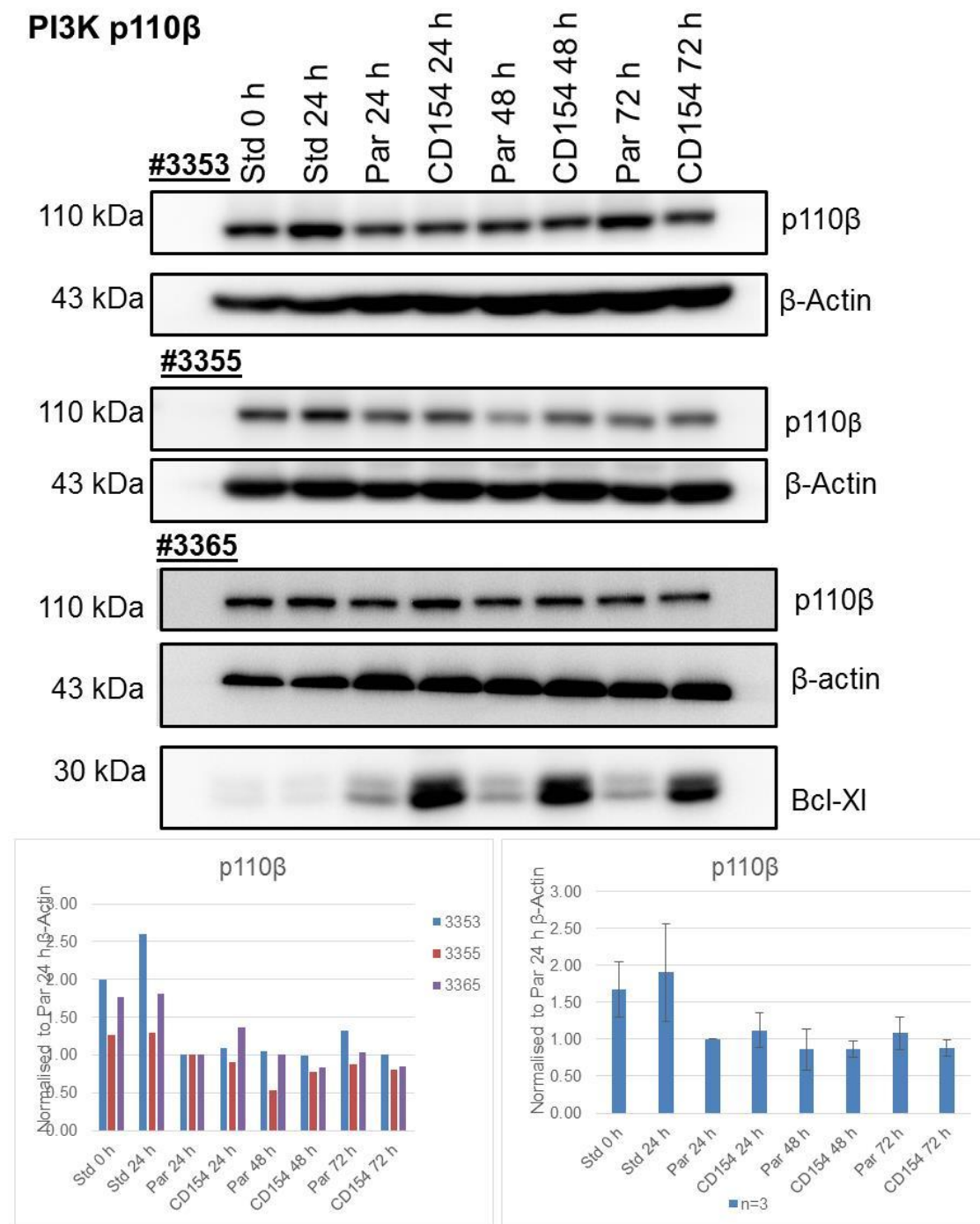


Figure 5.5: p110 β isoform expression upon CD40-stimulation of CLL cells.

CLL cells from three cases were thawed and recovered for 1 hour (Standard culture 0 h, Std 0 h), and cultured for 24 hours either alone (Standard culture 24 h, Std 24 h), co-cultured on parental fibroblasts (Par), or co-cultured with CD154-expressing fibroblasts (CD154) for 24 hours (24h), 48 hours (48h) or 72 hours (72h). Cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in modified RIPA with sonication, protein determination performed and 10 μ g of cell lysate loaded onto 10% SDS-PAGE gel. Proteins were transferred onto PVDF membrane. The membrane was incubated in primary antibodies overnight, followed by relevant HRP-conjugated secondary antibodies and the Western was developed using ECL on a CCD camera. Densitometry was performed using AIDA image analyser. β -Actin served as a loading control. Bcl-XL served as a positive control for CD154 stimulation. Graph shows the mean \pm SD. p-values were calculated using the student two-tailed paired t-test; p-values ≤ 0.05 are displayed.

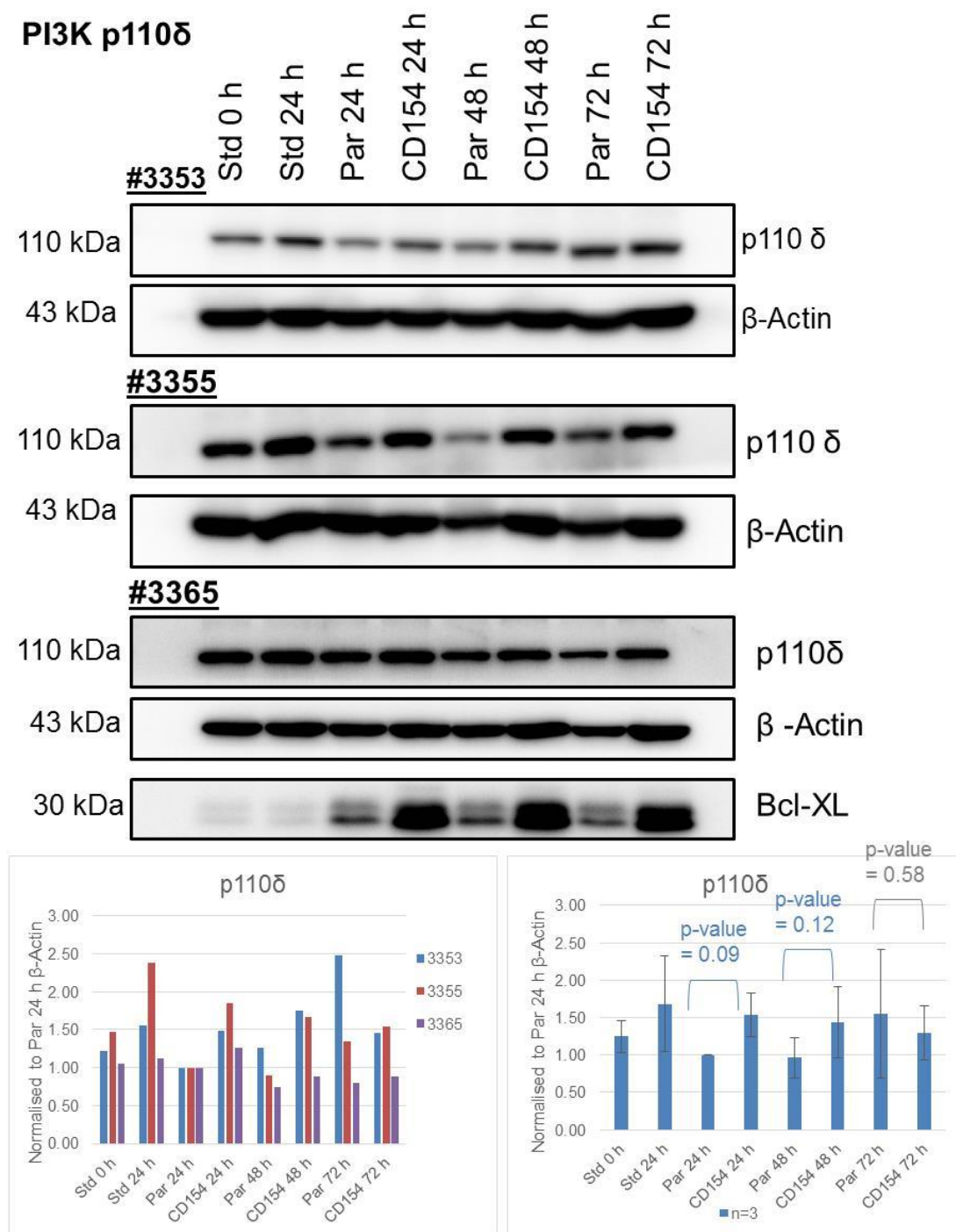


Figure 5.6: p110 δ isoform expression upon CD40-stimulation of CLL cells.

CLL cells from three cases were thawed and recovered for 1 hour (Standard culture 0 h, Std 0 h), and cultured for 24 hours either alone (Standard culture 24 h, Std 24 h), co-cultured on parental fibroblasts (Par), or co-cultured with CD154-expressing fibroblasts (CD154) for 24 hours (24h), 48 hours (48h) or 72 hours (72h). Cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in modified RIPA with sonication, protein determination performed and 10 μ g of cell lysate loaded onto 10% SDS-PAGE gel. Proteins were transferred onto PVDF membrane. The membrane was incubated in primary antibodies overnight, followed by relevant HRP-conjugated secondary antibodies and the Western was developed using ECL on a CCD camera. Densitometry was performed using AIDA image analyser. β -Actin served as a loading control. Bcl-XL served as a positive control for CD154 stimulation. Graph shows the mean \pm SD. p-values were calculated using the student two-tailed paired t-test; p-values are displayed.

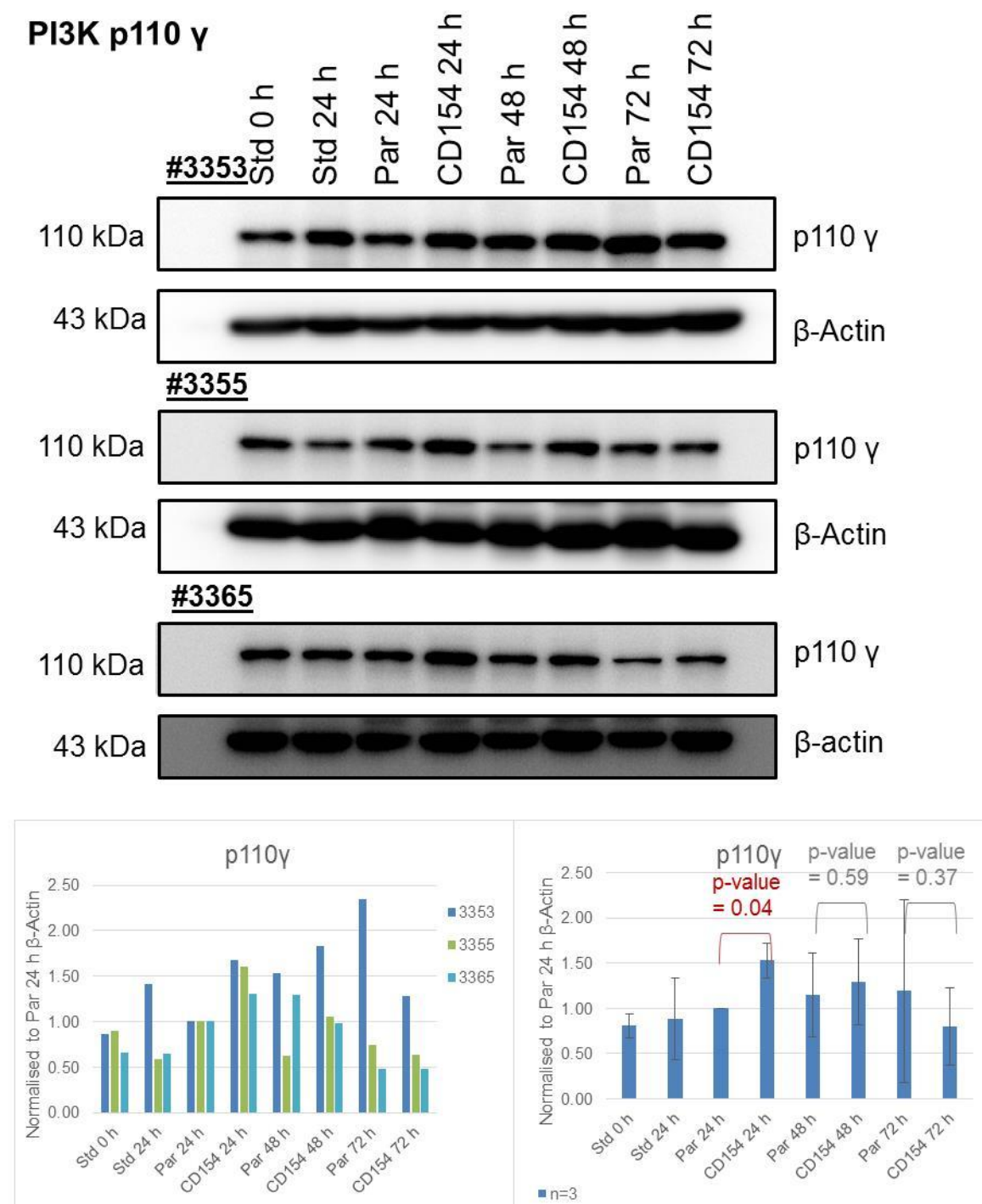


Figure 5.7: p110 γ isoform expression upon CD40-stimulation of CLL cells.

CLL cells from three cases were thawed and recovered for 1 hour (Standard culture 0 h, Std 0 h), and cultured for 24 hours either alone (Standard culture 24 h, Std 24 h), co-cultured on parental fibroblasts (Par), or co-cultured with CD154-expressing fibroblasts (CD154) for 24 hours (24h), 48 hours (48h) or 72 hours (72h). Cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in modified RIPA with sonication, protein determination performed and 10 μ g of cell lysate loaded onto 10% SDS-PAGE gel. Proteins were transferred onto PVDF membrane. The membrane was incubated in primary antibodies overnight, followed by relevant HRP-conjugated secondary antibodies and the Western was developed using ECL on a CCD camera. Densitometry was performed using AIDA image analyser. β -Actin served as a loading control. p-values were calculated using the student two-tailed paired t-test; p-values are displayed.

5.4 Regulation of AKT activation by the class I PI3K p110 isoforms in CD40-stimulated CLL cells

I showed in the previous chapter that phosphorylation of AKT was maintained in CLL cells upon CD40-stimulation ([Section 3.3](#)). I have also shown that CD40 stimulation increased the expression of PI3K p110 δ and γ isoforms. Since AKT is a major mediator of PI3Ks in regulating cell growth, proliferation and survival in most cell types including lymphocytes (Fruman et al., 2002, Fruman and Rommel, 2014, Manning and Cantley, 2007), I asked whether activation of AKT by CD40 stimulation was regulated by PI3K p110 δ and γ isoforms. To address this question, I used isoform-selective inhibitors and a pan-PI3K inhibitor (LY294002) that are all commercially available. The isoform specific inhibitors included GDC-0941 (PI3K p110 α/δ inhibitor) (Folkes et al., 2008), IPI-145 (PI3K p110 δ/γ inhibitor) (Winkler et al., 2013, Dong et al., 2014) and CAL-101 (Idelalisib, PI3K p110 δ inhibitor) (Lannutti et al., 2011). Given that both PI3K p110 δ and p110 γ isoforms were present in the CLL cells and their expression was increased by CD40 stimulation, I anticipated that IPI-145 would be more potent than CAL-101 at inhibiting CD40-mediated phosphorylation of AKT, since it is a more potent inhibitor than CAL-101 in inhibiting p110 δ , in addition to inhibiting p110 γ .

5.4.1 The effect of PI3K inhibitors on the viability of CD40-stimulated CLL cells

I first checked the cytotoxicity of various PI3K inhibitors on CLL cells co-cultured with either parental control or CD154-expressing fibroblasts. Primary CLL cells from three patient samples (#3347, #3354, #3357) were treated with the indicated concentrations of drugs and incubated on co-cultures for 24 hours. At the end of incubation, CLL cells were harvested and cell death assessed using a propidium iodide/flow cytometry method and the percentage of drug-induced cell death was calculated as described previously.

As shown in Figure 5.8A, all four PI3K inhibitors induced concentration-dependent cell death in CLL cells co-cultured with parental fibroblasts. LY294002 did not induce a significant amount of cell death at lower concentrations but caused about 50% cell death at 10 μ M concentration. Comparing to CAL-101, IPI-145 appeared to be more potent in inducing cell death at the range of concentrations examined. At 1 μ M concentration, IPI-145 is more cytotoxic than CAL-101 to CLL cells cultured on

CD154 fibroblasts (Figure 5.8B), suggesting that p110 γ may also be contributing to CLL survival. However, IPI-145 also has greater intrinsic potency against p110 δ than CAL-101 (Table 2.11 and Appendix Table 2), arguing for a greater role of p110 δ in CLL-cell survival. Cell death induced by IPI-145 and GDC-0941 at the range of concentrations tested was comparable regardless of whether CLL cells were co-cultured with parental or CD154-expressing fibroblasts (Figure 5.8A and B). Killing by CAL-101 and LY294002 at concentrations up to 10 μ M was slightly reduced in CLL cells co-cultured with CD154-expressing fibroblasts. At 10 μ M concentration, however, CAL-101 caused about 30% cell death in cells co-cultured with parental or CD154-expressing fibroblasts. Therefore, at the range of the concentrations used, IPI-145 or GDC-0941 are more potent than CAL-101 or LY294002 in inducing cell death of CD40-stimulated CLL cells.

n=3 #3347, #3354, #3357

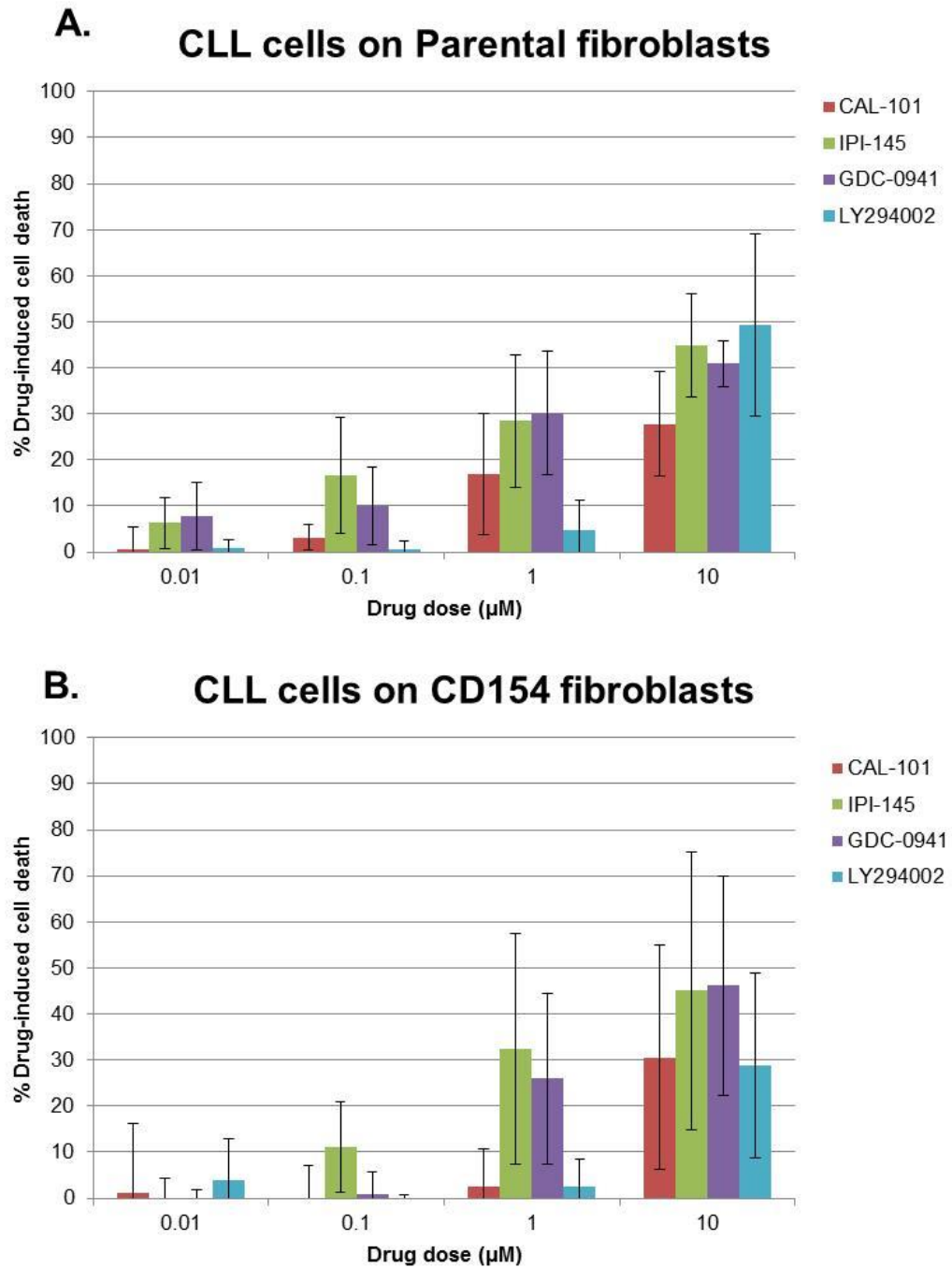


Figure 5.8: PI3K inhibitors induce cell death in CLL cells plated on parental and CD154-fibroblasts.

Three primary CLL cells (n=3 #3347, #3354, #3357) were recovered for 1 hour, and incubated with the indicated concentrations of drugs for 24 hours on either parental or CD154 fibroblasts. Cell death was assessed using propidium iodide/flow cytometry method. The percentage of drug-induced cell death was calculated as: $100 \times [(\% \text{ death of drug-treated cells} - \% \text{ death of untreated cells}) / (100 - \% \text{ death of untreated cells})]$.

- A. CLL cells plated on parental fibroblasts.
- B. CLL cells plated on CD154 fibroblasts.

5.4.2 The effect of PI3K p110 α / δ inhibitor on the phosphorylation of AKT in CD40-stimulated CLL cells

Next, I examined the effect of these PI3K inhibitors on the CD40-maintenance of AKT phosphorylation in CLL cells. CAL-101 has been shown to potently inhibit the phosphorylation of AKT in CLL cells when stimulated with soluble CD40 ligand (Herman et al., 2010), suggesting that PI3K p110 δ is involved in regulating CD40-mediated activation of AKT. I thus first tested whether the dual inhibition of PI3K p110 δ and α isoforms by GDC-0941 would have similar, if not greater, inhibition of AKT phosphorylation upon CD40 stimulation.

As shown in Figure 5.9, treatment with GDC-0941 of CLL cells co-cultured with CD154-expressing fibroblasts resulted in a concentration-dependent decrease in CD40-induced phosphorylation of AKT. GDC-0941 at 0.1 μ M reduced CD40-induced AKT phosphorylation by around 50% and at 1 μ M completely inhibited CD40-mediated phosphorylation of AKT. GDC-0941 at 10 μ M also reduced the level of phosphorylation of AKT below the level seen in untreated control cells.

Given that CAL-101 at 0.1 μ M concentration almost completely inhibited CD40-induced phosphorylation of AKT in CLL cells (Herman et al., 2010) whereas it required 1 μ M concentration for GDC-0941 to achieve similar inhibition as seen here, it appeared that additional inhibition of p110 α by GDC-0941 did not add any further reduction in AKT phosphorylation upon CD40 stimulation.

5.4.3 The effect of PI3K p110 δ / γ inhibitor on the phosphorylation of AKT in CD40-stimulated CLL cells

I next used IPI-145 to address the question of whether dual inhibition of PI3K p110 δ and γ would result in greater reduction in AKT phosphorylation in CLL cells upon CD40 stimulation. As shown in Figure 5.10, treatment with IPI-145 of CD40-stimulated CLL cells led to a concentration-dependent decrease in phosphorylation of AKT. IPI-145 at 0.01 μ M completely inhibited CD40-induced phosphorylation of AKT. This suggested that IPI-145 is more potent than CAL-101 in inhibiting AKT, due to either its greater intrinsic potency against p110 δ or the additional inhibition of PI3K p110 γ (Table 2.11 and Appendix Table 2).

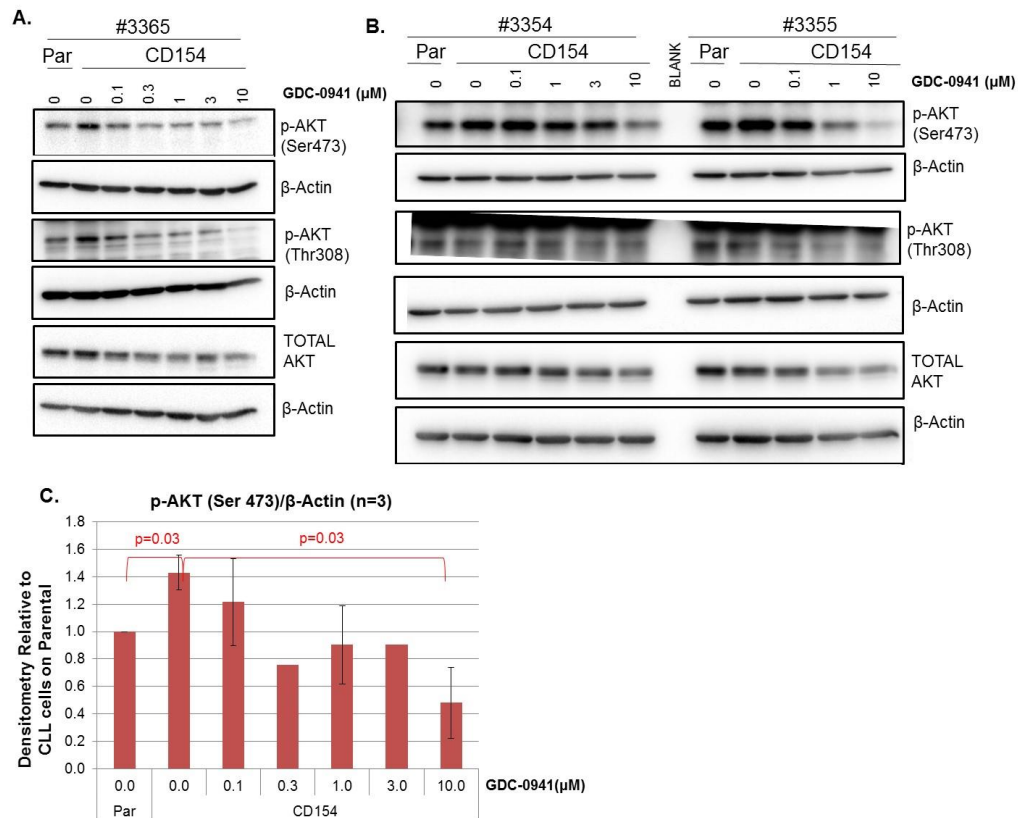


Figure 5.9: CD40-induced phosphorylation of AKT in CLL cells is inhibited by PI3K- α/δ inhibitor GDC-0941.

CLL cells from three cases were thawed and recovered for 1 hour and were co-cultured on parental fibroblasts (Par) or co-cultured with CD154-expressing fibroblasts (CD154) for 24 hours (24h), with the indicated doses of GDC-0941. Cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in modified RIPA with sonication, protein determination performed and 10 μ g of cell lysate loaded onto 10% SDS-PAGE gel. Proteins were transferred onto PVDF membrane. The membrane was incubated in primary antibodies overnight, followed by relevant HRP-conjugated secondary antibodies and the Western was developed using ECL on a CCD camera. Densitometry was performed using AIDA image analyser. β -Actin served as a loading control.

- A. Western blot of #3365 incubated with GDC-0941.
- B. Western blot of #3354 and #3355 incubated with GDC-0941.
- C. Densitometry of p-AKT(Ser473)/ β -Actin for n=3, paired two-tailed student T-TEST was performed. p-values ≤ 0.05 are displayed.

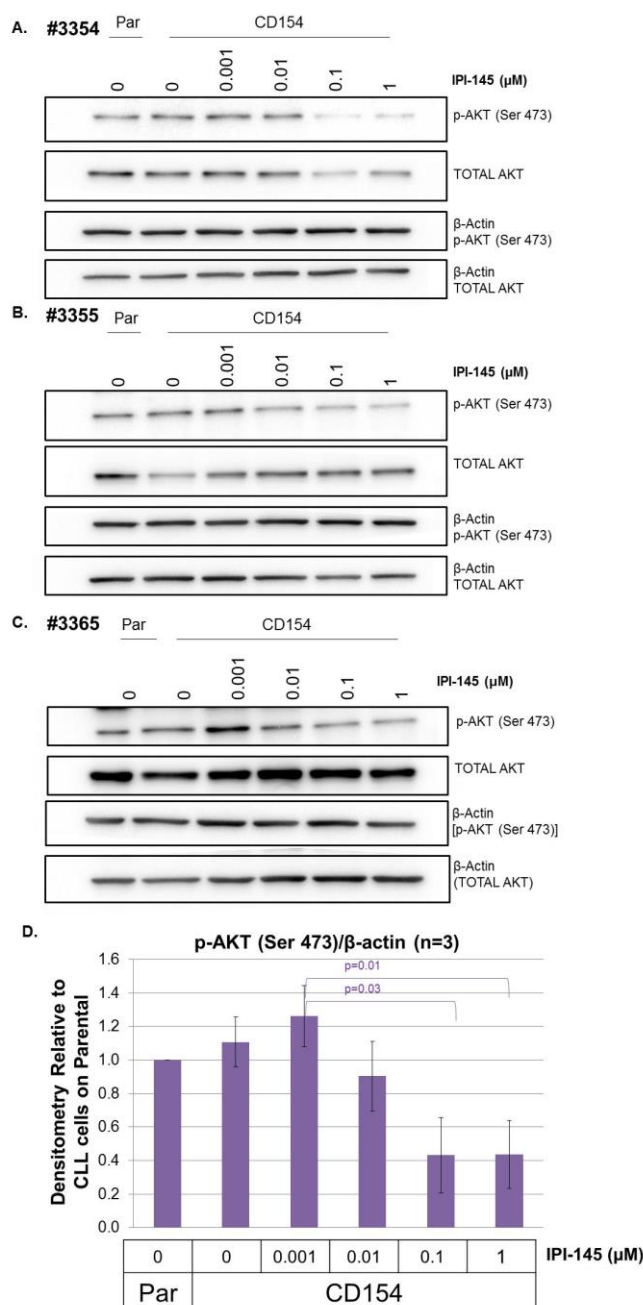


Figure 5.10: CD40-induced phosphorylation of AKT in CLL cells is inhibited by PI3K- δ/γ inhibitor IPI-145.

CLL cells from three cases were thawed and recovered for 1 hour and were co-cultured on parental fibroblasts (Par) or co-cultured with CD154-expressing fibroblasts (CD154) for 24 hours, with the indicated doses of IPI-145. Cells were harvested, washed twice with ice-cold PBS, and cell pellets frozen. Cell pellets were subsequently lysed in modified RIPA with sonication, protein determination performed and 10 μg of cell lysate loaded onto 10% SDS-PAGE gel. Proteins were transferred onto PVDF membrane. The membrane was incubated in primary antibodies overnight, followed by relevant HRP-conjugated secondary antibodies and the Western was developed using ECL on a CCD camera. Densitometry was performed using AIDA image analyser. β -Actin served as a loading control.

- Western blot of #3354.
- Western blot of #3355.
- Western blot of #3365.
- Densitometry of p-AKT(Ser473)/ β -Actin for n=3, paired two-tailed student T-TEST was performed. p-values ≤ 0.05 are displayed.

5.5 Summary

By comparing the protein expression of PI3K p110 catalytic subunits between CLL cells and normal B cells, I showed that PI3K p110 α was expressed at similar levels and PI3K p110 β at a lower level in CLL cells, whilst PI3K p110 δ and PI3K p110 γ were more abundantly expressed in CLL cells. A repeat experiment using purified CLL cells again showed that PI3K p110 γ was present in CLL cells and that the level was higher than in normal B cells.

The effect of CD40-stimulation on expression of PI3K p110 isoforms was also examined. CD40-stimulation decreased expression of PI3K p110 α whilst having no effect on the expression of PI3K p110 β in CLL cells. In contrast, both PI3K p110 δ and p110 γ isoforms were up-regulated in CD40-stimulated CLL cells.

Pan- and isoform-selective PI3K inhibitors were used to determine which PI3K p110 isoform maintains AKT activation in response to CD40 stimulation. Four PI3K inhibitors (CAL-101, IPI-145, GDC-0941 and LY294002) all induced concentration-dependent cell death in CLL cells co-cultured on either parental or CD154-expressing fibroblasts, indicating a pro-survival role of PI3K in CD40-stimulated CLL cells. Dual inhibition of PI3K p110 α and δ isoforms by GDC-0941 at 1 μ M completely inhibited CD40-induced phosphorylation of AKT. Inhibition of PI3K p110 δ and γ by IPI-145 at 0.01 μ M completely inhibited CD40-induced phosphorylation of AKT. Taken together, the data in this chapter suggests that the PI3K p110 δ isoform is mainly responsible for the maintenance of AKT in CLL cells in response to CD40 stimulation, but that the p110 γ isoform may also play a role.

5.6 Discussion

5.6.1 The expression of PI3K p110 isoforms in the CLL compared to normal B cells.

PI3K p110 α and PI3K p110 β are generally thought to be ubiquitously expressed, whereas PI3K p110 δ and PI3K p110 γ isoforms are considered to be preferentially expressed in leukocytes where they have distinct, non-overlapping roles in key cellular functions including cell proliferation, differentiation and migration ([Section 1.2.1.2](#)) (Kok et al., 2009, Vanhaesebroeck et al., 2010).

The PI3K p110 isoforms are known to play a vital role in the development, function and survival of B cells (Pauls et al., 2012, Werner et al., 2010, Baracho et al., 2011, Okkenhaug and Vanhaesebroeck, 2003) ([Section 1.2.1.3](#)). PI3K p110 δ is essential for B cell development, and for antigen receptor signalling in both B and T cells (Clayton et al., 2002, Jou et al., 2002, Okkenhaug et al., 2002). It has been estimated that p110 δ contributes to 60% of total PI3K activity in normal B cells whereas p110 α and β only represent the 40% of remaining activity (Bilancio et al., 2006). PI3K p110 δ has been shown to mediate IL-4 and BCR signalling in normal B cells (Bilancio et al., 2006). PI3K p110 α can compensate in the absence of p110 δ to promote early B cell development in the bone marrow and B cell survival in the spleen (Ramadani et al., 2010). PI3K p110 γ has also been implicated in early B cell development (Beer-Hammer et al., 2010). Mice double deficient in p110 γ /p110 δ had an increased percentage of pre-pro B cells and reduced splenic B cell numbers as compared with mice with a single deficiency, suggesting that p110 γ and p110 δ have overlapping roles in the development. Furthermore, B cells from these double deficient mice had reduced survival and were less responsive to proliferative stimuli such as LPS or CD40, suggesting p110 γ may also have a role in B cell proliferation and survival (Beer-Hammer et al., 2010).

There was limited information available in the literature on expression of PI3K p110 isoforms in CLL cells at the time of undertaking these experiments, except that PI3K p110 δ isoform had been shown to be abundantly expressed in CLL cells (Herman et al., 2010). Very recently, a study reported that primary CLL cells express abundant amounts of p110 δ and p110 β , but modest level of p110 γ and undetectable levels of p110 α although these observations were based on a small sample size (n=3) (Balakrishnan et al., 2015).

In my study, by comparing the levels of protein expression of PI3K p110 catalytic subunits between CLL cells and normal B cells, I showed that PI3K p110 α was expressed at similar levels in both cell types and PI3K p110 β expressed at a lower level in CLL cells, whilst PI3K p110 δ and p110 γ were abundantly expressed in CLL cells. A repeat experiment with purified CLL cells again showed that PI3K p110 γ was present in CLL cells and that the level of PI3K p110 γ was higher in CLL cells than normal B cells.

Thus my data confirmed the finding by Herman and colleagues that p110 δ isoform was abundantly expressed in CLL cells (Herman et al., 2010), but was in stark contrast to the result reported by Balakrishnan and colleagues that p110 α was undetectable and p110 γ expressed at modest level, whereas p110 β and p110 δ were abundantly expressed in CLL cells (Balakrishnan et al., 2015). The use of positive controls in the form of recombinant p110 α , β and δ proteins in my study convinced me that my results were reliable.

Previously, there was a suggestion in the literature that CLL cells from certain subgroups of CLL may express more PI3K than others. For example, expression of the *PI3K* gene at mRNA level was higher in UM-CLL than M-CLL (1.7 fold increase) (Kienle et al., 2006) and expression of PI3K protein was also 1.5-fold higher in the normal karyotype of CLL (Winkler et al., 2010). Of the ten CLL samples that I have studied for p110 isoform expression, two samples were of normal karyotype (#2814, #2441), two samples carried deletions in chromosome 17p (#2674, #2103), two samples carried deletions in chromosome 11q (#2474, #2263), two samples carried deletions in chromosome 13q (#3074, #1958), and two samples had trisomy 12 (#2064, #2521). Cell lysates prepared from CLL cells obtained from patients with these different genetic backgrounds were examined for the expression of PI3K p110 isoforms on the same blots by Western blotting. There did not appear to be any consistent pattern in expression of these isoforms correlating with a particular genetic background. The cells with no FISH defects certainly did not appear to have 1.5-fold more p110 isoforms than other genetic subtypes. Whilst this is a small sample size, I found no indication that any genetic background is more likely to have increased PI3K p110 levels than another. Regarding *IGHV* mutational status, two samples were mutated (#1958 and #2521), whereas eight samples were unmutated. However, there did not appear to be reduced PI3K p110 expression in the two mutated samples.

It is worth noting that the normal B cells used were not age-matched, since they were collected from healthy volunteers who are younger than the average CLL patient. However, there is no evidence to suggest that PI3K isoform expression levels should vary with age.

The finding that PI3K p110 β was expressed at a lower level in CLL cells than normal B cells is novel. PI3K p110 δ had previously been shown to be abundantly expressed in CD19⁺ cells from twenty patients with CLL, but the level of its expression did not vary among the patient samples, although it did appear to be slightly higher in CLL patient cells than normal B cells (Herman et al., 2010). My results are in agreement with the above findings regarding the expression of PI3K p110 δ in CLL cells.

The finding that PI3K p110 γ was present in CLL cells was novel. To confirm that PI3K p110 γ was indeed expressed in the CLL B cells, I enriched CLL B cells using a negative selection method and obtained greater than 97% purity as measured by double positivity for CD5 and CD19. Therefore, I was convinced that CLL cells express PI3K p110 γ . However, there is still a possibility that PI3K p110 γ is from the 3% contaminating dendritic cells, NK cells or myeloid cells. Further work is thus needed, and currently being undertaken within the Liverpool CLL group, to confirm that PI3K p110 γ is indeed present in CLL B cells.

I did not examine PI3K enzymatic activity in my study. Herman and colleagues added whole-cell extracts to a mixture of PIP₂ and ATP and used an ELISA method to measure PI3K enzyme activity. They demonstrated that CD19⁺ CLL cells overall had a significantly higher PI3K enzymatic activity than normal B cells (Herman et al., 2010). It is, however, unclear whether this higher PI3K enzymatic activity is largely due to PI3K p110 δ or whether PI3K p110 α or p110 γ have a role to play.

5.6.2 Regulation of expression of PI3K p110 isoforms by the CLL microenvironment

I was interested in examining the expression of PI3K p110 isoforms in the CLL microenvironment. Previous work showed that ‘co-culturing CLL primary cells with bone marrow stromal cells to mimic the leukaemic microenvironment increased the protein levels of all four Class I PI3K isoforms’ (Balakrishnan, 2013). However it appears that the effect of CD40 on p110 isoforms is specific.

The CD40 stimulation data along with the expression in unstimulated CLL cells compared to normal B cells suggests that PI3K p110 δ and p110 γ isoforms are significant in CLL pathophysiology, particularly in the context of the CLL

microenvironment. PI3K p110 α , whilst abundantly expressed in CLL cells from PBMCs, is not upregulated in CLL cells upon CD40-stimulation and does not seem to mediate microenvironment signalling. The expression of PI3K p110 β did not change upon CD40 stimulation, indicating that p110 β may not be important in mediating signals in the CLL microenvironment. However, it should be noted that expression may not be as important as activity. In order to fully understand the contribution each p110 isoform makes to overall PI3K activity in CLL cells, experiments where individual p110 isoforms are knocked down would ideally be performed. Better still, experiments where individual isoforms are knocked down followed by the expression of a kinase dead version of the same isoform would be useful. However, achieving knockdown of genes in CLL cells is difficult (see [section 6.4.3](#)).

Exactly how or why PI3K p110 δ and p110 γ are upregulated upon CD40 stimulation is currently unknown. CD40 stimulation has been shown to increase PI3K p110 δ activity as measured by p-AKT (Ser473) (Herman et al., 2010). Whether CD40 stimulation increases PI3K p110 γ activity is unclear. However, as described earlier animal studies showed that p110 γ and p110 δ have some overlapping functions in response to CD40 stimulation (Beer-Hammer et al., 2010). How this is possible when the current models for p110 γ and p110 δ activation are different is unclear. Current understanding is that p110 γ is activated by engagement of GPCRs via p101, whereas p110 δ is activated by RTKs via p85. However, whether the upregulation of PI3K p110 δ or p110 γ upon CD40 stimulation is mediated at the transcriptional or post-transcriptional level is not clear. Promoter analysis of *PIK3CG* revealed binding sites for transcriptional factors c/EBP β and GATA-1 (Kok et al., 2009). However, whether CD40 stimulation results in increased activity of c/EBP β and GATA-1 is unknown.

5.6.3 Inhibition of PI3K p110 isoforms in CD40-stimulated CLL cells

I used pan- and isoform-selective PI3K inhibitors to test which PI3K p110 isoform maintains CD40-induced AKT activation. Understanding the biology of CD40 signalling may allow for therapeutic intervention, using inhibitors to antagonise the protective effect of CD154 on CLL cells. Previously, Herman and colleagues stimulated CD19⁺ CLL cells from three patients with 1 μ g/mL soluble CD40L for 2

hours in the presence or absence of various concentrations of CAL-101 (a PI3K p110 δ inhibitor) and monitored AKT phosphorylation at S473 by Western blotting. Stimulation of CLL cells with soluble CD40L increased AKT phosphorylation by about 1.5-fold and addition of 0.1 μ M CAL-101 reduced this phosphorylation back to basal levels. Higher doses of CAL-101 at 1 μ M and 10 μ M further reduced this phosphorylation below the level of p-AKT in the untreated cells (Herman et al., 2010).

In my study, all four PI3K inhibitors (CAL-101, IPI-145, GDC-0941 and LY294002) induced concentration-dependent cell death in CLL cells co-cultured with either parental or CD154-expressing fibroblasts. I also observed that any protective effect in CLL cells upon CD40 stimulation was ablated by the four PI3K inhibitors I used. This data is in keeping with previously published results showing that CAL-101 induced time- and dose-dependent cell death in CLL B cells in standard culture conditions and CAL-101 also abrogated the protective effect provided by CD40L (Herman et al., 2010).

The dual inhibition of PI3K p110 δ and α isoforms by GDC-0941 at 1 μ M completely inhibited CD40-maintained phosphorylation of AKT. Ideally, direct comparison between CAL-101 and GDC-0941 should have been used in these experiments; however, CAL-101 was not used in these experiments because of limited amount of primary CLL cells available to work with (See [section 6.4.4](#) for more information). Given that CAL-101 at 0.1 μ M concentration almost completely inhibited soluble CD40-induced phosphorylation of AKT in CLL cells (Herman et al., 2010) whereas in my experiments it required 1 μ M concentration for GDC-0941 to achieve similar inhibition, it appeared that additional inhibition of p110 α by GDC-0941 did not add any further reduction in AKT phosphorylation upon CD40 stimulation.

Inhibition of PI3K p110 δ and γ by IPI-145 at 0.01 μ M completely inhibited CD40-maintained phosphorylation of AKT. This result is similar to that reported by Dong and colleagues who examined the effect of IPI-145 on AKT phosphorylation induced by BCR cross-linking. BCR cross-linking caused a 1.5-fold increase in phosphorylation of AKT (S473), which was significantly inhibited by IPI-145 at a concentration as low as 0.01 μ M (Dong et al., 2014). These results suggest that either the greater intrinsic potency against p110 δ or the additional inhibition of PI3K p110 γ

by IPI-145 further decreases AKT phosphorylation in CLL cells, as compared to inhibition of PI3K p110 δ alone by CAL-101 after stimulation with soluble CD40L (Herman et al., 2010). An inhibitor specific to p110 γ , or siRNA (or lentivirus expressing shRNA) to p110 δ and p110 γ would be useful to deconvolute the role of p110 δ and p110 γ further.

Collectively, the data in this chapter suggest that PI3K p110 γ may also play a role in the signalling to AKT in CLL cells, and that agents such as IPI-145 with additional p110 γ pharmacology may potentially have more therapeutic benefit than CAL-101, which is p110 δ selective.

Chapter 6 : General discussion

6.1 Role of AKT in CLL-cell survival

In the first part of my PhD study I sought to establish whether AKT was required for CLL-cell survival, particularly in a context of modelled lymph node microenvironment.

Previous studies had suggested that AKT was vital for CLL-cell survival in response to BCR stimulation (Bernal et al., 2001, Petlickovski et al., 2005, Longo et al., 2008). Other studies suggested that AKT was vital for CLL-cell survival in unstimulated cells since pharmacological inhibition of AKT (A-443654 and AKTi-1/2) or knockdown of AKT1 using siRNA led to increased cell death in CLL cells (Zhuang et al., 2010, de Frias et al., 2009, Hofbauer et al., 2015). Despite reports that stimulation of CLL cells by soluble CD40 ligand or anti-CD40 antibodies led to AKT activation (Cuni et al., 2004, Herman et al., 2010), the role of AKT in CLL cells co-cultured with CD154-expressing fibroblasts, a model system that mimics interactions between T cells and CLL cells through CD154-CD40 signalling pathway in the lymph node microenvironment had not been examined.

I hypothesised that AKT plays an important role in mediating survival signals in the CLL microenvironment and inhibiting AKT using the novel AKT inhibitor AZD5363, either alone or in combination with chemotherapeutic agents currently in use in clinic, would lead to increased cell death in CD40-stimulated CLL cells. To this end, in the study I used a novel ATP-competitive and relatively specific inhibitor of all three isoforms of AKT, AZD5363 (Davies et al., 2012, Banerji, 2013).

Consequently, I found that, the AKT inhibitor, AZD5363, is active and inhibits AKT in CLL cells. However, AZD5363 at the concentrations (3-10 μ M) that inhibit AKT was not cytotoxic since AZD5363 at these concentrations had no effect on cell viability of CLL cells after 24 h culture under standard conditions. AZD5363 did not sensitize un-stimulated CLL cells to bendamustine-induced killing. These initial results suggested that AKT was not vital for CLL-cell survival in unstimulated cells.

The above result was in stark contrast to the previously reported findings that AKT was vital for CLL-cell survival since pharmacological inhibition of AKT (A-443654 and AKTi-1/2) induced extensive cell death at concentrations close to the respective

IC₅₀ values in inhibiting AKT activity in CLL cells (Zhuang et al., 2010, de Frias et al., 2009, Hofbauer et al., 2015). This discrepancy, regarding the effect of AKT inhibitors on the survival of CLL cells cultured under standard conditions may in part be explained by A-443654 and AKTi-1/2 having many off-target effects (Bain et al., 2007). A-443654 and Akti-1/2 might inhibit enzymes other than AKT that are important for the survival of CLL cells cultured under standard conditions.

However, knockdown of AKT1 using siRNA had also previously been shown to induce cell death in unstimulated cells (Zhuang et al., 2010, Hofbauer et al., 2015). Since siRNA is usually more specific and has few off-target effects than the chemical inhibitors, this also contradicts with my interpretation based on the data obtained from experiments with AZD5363 that AKT was not vital for CLL cell survival in unstimulated cells. How could I reconcile these differences? It is possible that AZD5363 might inhibit not only AKT-mediated pro-survival signals but also AKT-independent death signals with a neutral net effect on CLL-cell survival under standard culture conditions. Regarding the specificity of AZD5363, it was noted that the compound inhibited P70S6K and PKA with an IC₅₀ similar to that observed for AKT2 and AKT3 (approximately 7 nM) and other enzymes (ROCK2, MKK1, MSK1, MSK2, PKC γ , PKG α , PKG β , PRKX, RSK2, and RSK3) with an IC₅₀ of less than 1 μ M in *in-vitro* cell-free assays (Davies et al., 2012). It is unclear whether incubation of intact cells with 10 μ M AZD5363 would achieve intracellular drug concentration close to 10 μ M. However, it seems plausible that AZD5363 might also have some “off-target” effects, which may explain differences in functional outcomes observed.

In addition, Ding and colleagues showed that MK-2206, an allosteric AKT inhibitor which binds AKT at PH-domain, was able to induce time-dependent apoptosis in primary CLL cells from 12 patients (Ding et al., 2013). However, the concentration of MK-2206 (8 μ M) needed to induce 50% apoptosis in CLL cells over 72 hours is eight-fold higher than the concentration (1 μ M) required to inhibit phosphorylation of AKT at serine 473. The possibility that cell death induced by the higher concentration of MK-2206 was due to off-target effects cannot be excluded. Whilst relative selectivity of MK-2206 for AKT has been tested against 250 other kinases, the fact that MK-2206 may also bind to other PH or PX-domain containing proteins

such as BTK or SGK3, especially at high concentrations suggests that it could inhibit other kinases that are critically involved in the survival of CLL cells.

Last but not least, the difference might be explained by a recent report of a kinase-independent function of AKT that also promotes cancer cell survival (Vivanco et al., 2014) (Figure 6.1). Vivanco and colleagues also observed that ATP-competitive and allosteric AKT inhibitors had differing effects on cell survival. They showed that inhibiting the kinase activity of AKT did not inhibit PH-domain-mediated cell survival, nor did it increase drug-induced cell death in human lung and breast cancer cells (Vivanco et al., 2014). They showed that the greater cell death induction by the allosteric AKT inhibitor was indeed due to specific binding of MK-2206 to an amino acid within PH domain of AKT as mutation of this residue (W80A in AKT1 or AKT2) interrupted the binding and abolished MK-2206-induced cell death. Vivanco and colleagues speculated that the PH-domain promotes cell survival through regulation of protein-protein interaction (Vivanco et al., 2014). They suggest further studies are required to identify the AKT interacting proteins and to subsequently determine their contribution to AKT regulated survival (Vivanco et al., 2014). Therefore, the cytotoxicity resulting from prevention of recruitment of AKT to the plasma membrane by MK-2206 or knockdown of AKT1 by siRNA, which was not observed with AZD5363, might explain these differences in toxicity (Figure 6.1).

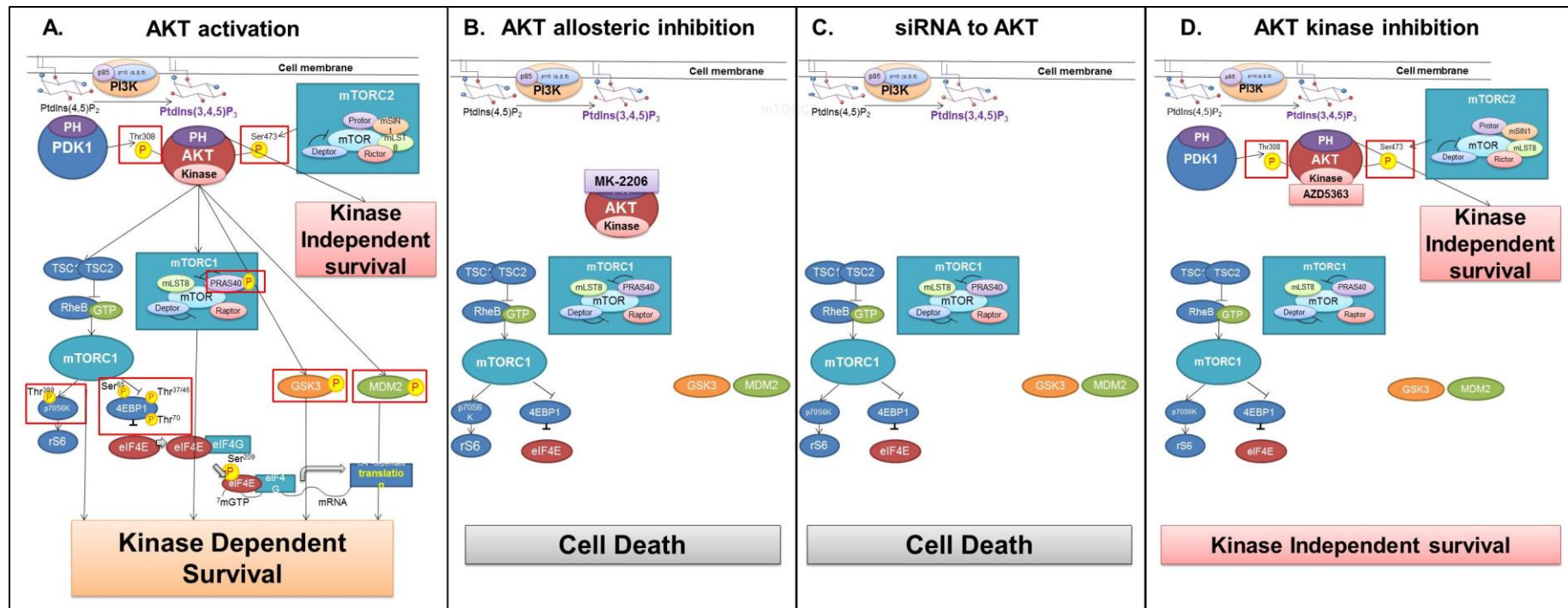


Figure 6.1: Two modes of action of AKT inhibitors: allosteric inhibition and kinase inhibition. Figure by Elinor Chapman.

- AKT activation occurs when Class I PI3Ks phosphorylate PtdIns(4,5)P₂ to generate PtdIns(3,4,5)P₃. The pleckstrin homology (PH) domain of AKT binds to PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ driving translocation of AKT to the plasma membrane and allowing its phosphorylation on Thr308 by phosphoinositide-dependent kinase 1 (PDK1). Additional phosphorylation on Ser473 by mammalian target of rapamycin complex 2 (mTORC2) leads to full activation. Once fully active, AKT drives phosphorylation of a plethora of downstream protein targets, a selection of which is shown, including indirect activation of mTORC1, direct phosphorylation of PRAS40 (a subunit of mTORC1), direct phosphorylation of GSK3 and MDM2 and other substrates, leading to kinase dependent survival (orange box). There also appears to be a kinase independent mechanism of survival (red box).
- Allosteric inhibition of AKT, by MK-2206 binding the PH-domain, prevents AKT translocating to the plasma membrane. No downstream targets are phosphorylated. Since there are no survival signals, cell death occurs.
- siRNA diminishes expression of AKT. Since there are no survival signals, cell death occurs.
- Kinase inhibition of AKT, via AZD5363 binding the ATP-binding site, results in AKT still being recruited to the plasma membrane, and AKT being hyper-phosphorylated, whilst the downstream substrates are not phosphorylated. But there is some kinase independent survival.

I showed that AKT activation is maintained in CLL cells following CD40 stimulation. I also showed that the AKT inhibitor, AZD5363 (10 μ M), is still active in inhibiting AKT in CD40-stimulated CLL cells. Furthermore, I showed that AZD5363 (10 μ M) reduced the viability of CLL cells co-cultured with CD154-expressing fibroblasts and sensitized these CD40-stimulated cells to bendamustine-induced killing. Addition of AZD5363 also increased cell death induced by 100 μ M bendamustine in CD40-stimulated CLL cells with chromosomal deletion in 17p, even though these cells are less sensitive to bendamustine (100 μ M) alone. This suggests that the sensitising effect of AZD5363 to bendamustine does not involve p53 since these cells do not express p53. Taken together, my results suggest that AKT plays a role in mediating the protective effect of CD40 stimulation against drug (bendamustine) induced cell death. Pharmacological inhibition of AKT may therefore have therapeutic potential, especially in eliminating tissue resident CLL cells in, for example, the lymph nodes. To my knowledge, this is the first time that the effect of AKT inhibition in CD40 stimulated CLL cells has been examined.

6.1.1 Effect of PI3K inhibition on survival of CLL cells

In *in vitro* studies, both pan- and isoform-specific PI3K inhibitors have been shown kill CLL cells in a dose- and time-dependent fashion. A pan PI3K inhibitor, BKM120 (Amrein et al., 2013), and PI3K- α/δ inhibitor, copanlisib (BAY 80-6946) induced CLL-cell death (Gockeritz et al., 2015). The PI3K p110 δ -selective inhibitor, idelalisib and the PI3K p110 δ/γ inhibitor, duvelisib, also killed cells in a dose- and time-dependent (Herman et al., 2010, Balakrishnan et al., 2015). It has also been shown that inhibition of p110 α , β and δ (using isoform selective class I PI3K inhibitors) induced apoptosis whereas inhibition of p110 γ did not affect the viability of CLL cells, suggesting that p110 α , β and δ but not γ contribute to survival of CLL cells (de Frias et al., 2010).

Indeed my experiments using four different PI3K inhibitors (CAL-101, IPI-145, GDC-0941 and LY294002) showed that they all induced concentration-dependent killing of CLL cells co-cultured on either parental or CD154-expressing fibroblasts ([Section 5.4.1](#), Figure 5.8). In keeping with the results reported by Herman and colleagues, I found that CAL-101 and LY294002 were not significantly different in their cytotoxicity towards CLL cells (Herman et al., 2010). I didn't examine the

cytotoxicity of PI3K inhibitors on other haematological cells. However, Dong and colleagues and Herman and colleagues found that B cells were more sensitive to CAL-101 and IPI-145 than T and NK cells in CLL patients (Dong et al., 2014).

Taken together, this evidence is overwhelming that PI3K plays a critical role in the survival of CLL cells and pharmacological inhibition of PI3K has therapeutic benefit. Given that AKT is an important kinase downstream of PI3K, I speculated that targeting AKT may have similar effects. However, although inhibiting AKT by AZD5363 (10 μ M) induced cell death in CLL cells co-cultured with CD154-expressing fibroblasts, the extent of death was much less than that induced by PI3K inhibitors. This suggests that other pro-survival kinases/molecules may also be inhibited by PI3K inhibitors. Indeed, it has been shown that cell death induced by PI3K inhibitors in some leukaemic cells was mediated through inhibition of MAPK pathway rather than AKT inhibition (Rahmani et al., 2003).

6.2 AKT and PI3K in CLL-cell proliferation

CLL is a proliferative disease (Messmer et al., 2005). CLL-cell proliferation occurs in pseudofollicles of the lymph node (van Gent et al., 2008, Herishanu et al., 2011) where CLL cells attract and interact with CD4⁺ and CD154⁺ T cells (Ghia et al., 2002, Schmid and Isaacson, 1994, Granziero, 2001, Tretter et al., 1998). This interaction between T cells and CLL cells, through engagement of CD40 on CLL cells by its cognate ligand CD154, leads to CLL-cell proliferation (Patten et al., 2008, Bagnara et al., 2011, Os et al., 2013). Having shown that AKT plays a role in mediating survival signals in CLL cells, in response to CD40-stimulation I next sought to examine whether AKT was required in CD40 stimulated CLL cell growth, cell cycle progression and proliferation.

6.2.1 Role of AKT in CLL-cell growth

As described earlier, AKT promotes cell growth, cell cycle progression and proliferation in most, if not all, all cell types in response to growth factors. AKT mediates cell growth and protein synthesis via mTORC1 and its downstream targets S6K1 and 4EBP1/eIF4E resulting in increased cell size (Edinger and Thompson, 2002, Fingar et al., 2002). AKT may also regulate cell size through other pathways, such as GSK3 (Gingras et al., 1998) ([Section 1.3.4.3](#)).

Longo and colleagues showed that constitutive activation of AKT increased the size of CLL cells (Longo et al., 2008). Since CD40 stimulation of CLL cells over five days also resulted in an increase in size (Cuni et al., 2004), I reasoned that inhibiting AKT activity would reduce CD40-stimulated increase in cell size. To test this, I employed two AKT inhibitors with differing modes of action, namely AZD5363 and MK-2206. Both inhibitors were shown to reduce cell size increase in CLL cells co-cultured with CD154-expressing fibroblasts in the presence of either IL-4 or IL-21, suggesting a role for AKT in CLL-cell growth.

Inhibition of AKT did not significantly inhibit the increase in size of normal B cells in response to CD40 + IL-4 stimulation, albeit based on the small sample size (n=3). AKT inhibition did reduce the growth of CD40 + IL-21-stimulated normal B cells. Therefore, these results suggest a role of AKT in growth of normal B cells in response to CD40 + IL-21 stimulation.

Whilst inhibition of AKT certainly decreased CD40-induced CLL-cell growth, it did not totally inhibit CD40-induced cell growth, suggesting that other pathways are also involved. For example the pro-survival NF- κ B signalling pathway has been known to be activated following CD40 stimulation in CLL cells (Schattner, 2000, Furman et al., 2000). NF- κ B signalling plays a critical role in tumour cell growth and proliferation (Karin, 2006, Hoesel and Schmid, 2013).

6.2.2 Role of AKT in CLL-cell division

I showed that AZD5363 inhibited CD40 + IL-4 induced proliferation in CLL-cells but not in normal B cells. This suggested a selective requirement of AKT for CD40 + IL-4-induced proliferation in CLL cells. Two AKT inhibitors, with different modes of action, inhibited CD40 + IL-21-induced proliferation in CLL cells from three out of five of the patient samples, but failed to inhibit such proliferation in CLL cells from two other patient samples. Neither AZD5363 nor MK-2206 inhibited the proliferation of normal B cells induced by CD40 + IL-21 stimulation. AKT is therefore required for CD40 + IL-21-induced proliferation in CLL cells from some but not all patient samples, and not required for such proliferation in normal B cells.

In one CLL sample (#2814) in which proliferation induced by CD40 + IL-4 was inhibited by AZD5363, proliferation induced by CD40 + IL-21 was not inhibited by

either AZD5363 or MK-2206. This implies that CD154 + IL-21-induced proliferation is less dependent upon AKT. As far as the activated transcription factors are concerned, IL-4 appears to selectively activate STAT6 following its phosphorylation upon IL-4 stimulation (Wick and Berton, 2000, Takeda et al., 1996) whereas IL-21 induces phosphorylation of STAT3 (Pascutti et al., 2013).

In the two CLL samples where AKT inhibitors did not inhibit CD40 + IL-21-induced proliferation, AZD5363 (1, 3, 10 μ M) and MK-2206 (1 μ M) caused an increase in proliferation of CLL cells. CLL cells from these two cases also seemed to be most sensitive to the highest dose of MK-2206 (10 μ M), which caused significant cell death as measured by PI/flow cytometry method. However, I am currently unable to explain why inhibition of AKT leads to increased proliferation induced by CD40 + IL-21 stimulation in these CLL cells.

6.2.3 Role of AKT in regulating cell cycle-regulatory molecules

In order to understand the role of AKT in regulating the cell cycle in CD40 + IL-21-induced proliferation, I examined the alterations in cell cycle-related molecules including some cyclins, CDKs and CDKis in CD154 + IL-21-stimulated CLL cells in the presence of the AKT inhibitor, AZD5363. In keeping with the literature, I showed that unstimulated primary CLL cells express hardly any cyclins or CDKs (Vrhovac et al., 1998, Korz et al., 2002, Decker et al., 2002). Instead, unstimulated cells express high levels of the CDKi p27, but not p21.

In CLL cells co-cultured with CD154-expressing fibroblasts + IL-21, expression of p27 rapidly decreased whereas expression of cyclins D2 and D3, and CDKs 2 and 4 significantly increased (day 1). Cyclins A2 and E1, and CDK1 were also induced by stimulation with CD40 + IL-21 for two to three days. Inhibition of AKT by AZD5363 inhibited induction of cyclins A2, D2 and D3, and CDKs1 and 4. Since cyclin A2 and CDK1 are required for the G2/M transition of the cell cycle, this suggests that AKT is also critically involved in the later phase of the cell cycle progression of CLL cells stimulated by CD40 + IL-21.

My data described above are thus in agreement with findings on the role of AKT in cell cycle progression of CLL cells in response to CpG-ODN stimulation (Longo et al., 2007). In their study, Longo and colleagues have shown that the differential

capacity of CLL cells to undergo CpG-ODN-induced cell-cycle progression and proliferate is largely dependent on AKT. Those CLL cells that were responsive to CpG-ODN-induced proliferation had higher levels of phosphorylated AKT and were able to induce cyclin A. Enforced expression of constitutively active AKT in CLL cells that were non-responsive to CpG-ODN-induced proliferation resulted in the induction of cyclin A and cell cycle progression in response to CpG-ODN stimulation. They also demonstrated a correlation between activation of AKT and expression of cyclin A.

In addition, it has also been shown that AKT activity fluctuates across the different phases of the cell cycle, mirroring the expression of cyclin A2 (Liu et al., 2014). Liu and colleagues thus showed that deletion of cyclin A2 led to reduced AKT activity and increased apoptosis, and that enforced expression of active AKT1 can rescue cyclin A2 deletion-induced apoptosis in mouse embryonic stem cells. It is therefore possible that AKT activity may also be linked to the expression of cyclin A2 in CLL cells.

I have created a model of CD40-induced proliferation as depicted in Figure 6.2, CD40 stimulation also results in the activation of NF κ B, JNK, p38 and PI3K, as well as JAKs. Cytokines IL-4 and IL-21 signal via their respective receptors. IL-4 results in the phosphorylation of STAT6, whereas IL-21 results in the phosphorylation of STAT3. Recently it has been shown that CD40 stimulation can induce the upregulation of miR-22, which targets PTEN for degradation, resulting in an increase in AKT activity. AKT phosphorylates FOXOs and many other substrates. FOXOs normally acts as transcription factors that activate expression of genes required for cell cycle arrest, e.g. p27, and induction of apoptosis, such as BIM. Phosphorylation of FOXOs by AKT causes them to relocate from the nucleus to the cytoplasm where they are subjected to proteosomal degradation, thereby allowing the cell cycle to progress. CD40 stimulation + IL-4 results in modest proliferation. CD40 stimulation + IL-21 results in more profound proliferation. In my study, AKT inhibitors, MK-2206 and AZD5363 were shown to inhibit CD154 + IL-4-induced proliferation by 50%, and to inhibit CD154 + IL-21-induced proliferation in 3/5 cases tested (Figure 6.2).

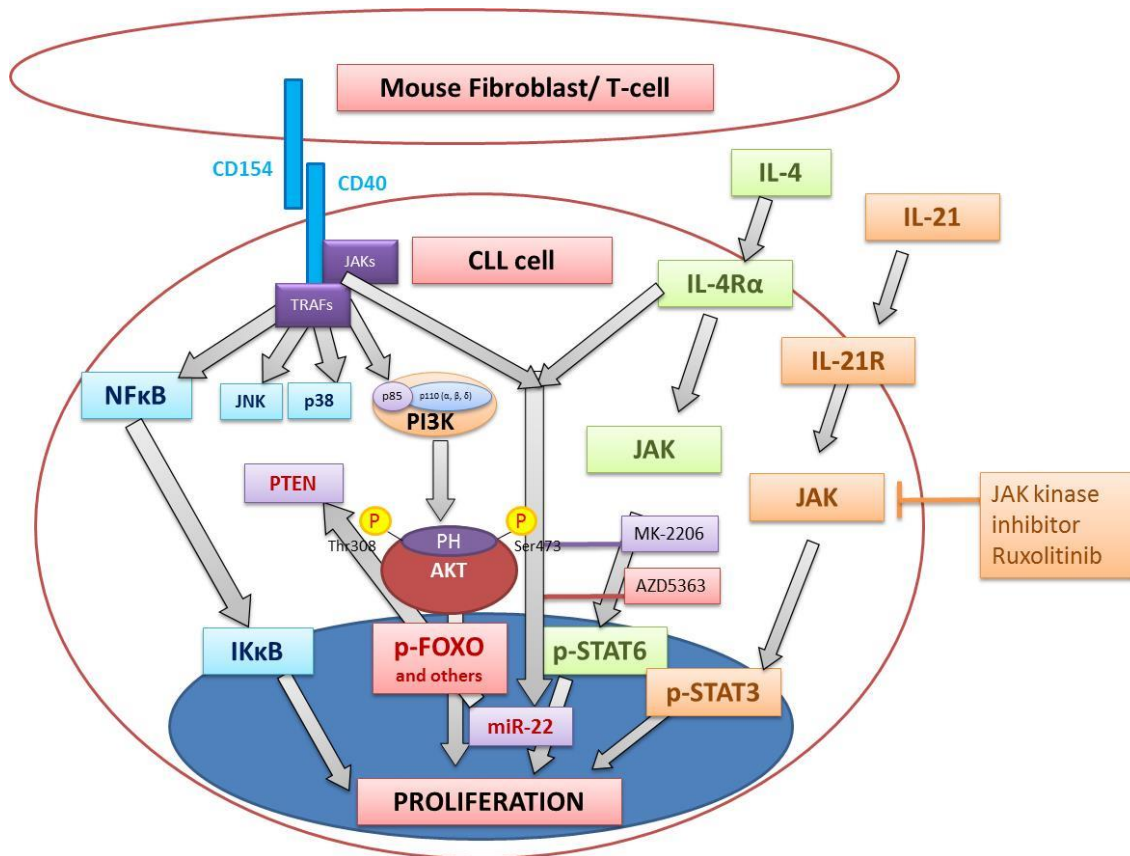


Figure 6.2: Diagram of how CD40 stimulation plus cytokines might result in proliferation of CLL cells.

CD154 expressed on T-cells, or in the case of the in vitro model, on mouse fibroblasts interacts with CD40 on CLL cells. This interaction is thought to be trimeric (not shown in diagram). CD40 stimulation results in the activation of NFκB, JNK, p38 and PI3K, as well as JAKs. There is some evidence that activation of AKT signalling pathway has been documented to be dependent upon CD40 lipid raft association, which is independent of any signalling events (Nadiri et al., 2011). Cytokines IL-4 and IL-21 signal via their respective receptors. IL-4 results in the phosphorylation of STAT6, whereas IL-21 results in the phosphorylation of STAT3. CD154 + IL-4 stimulation leads to the upregulation of miR-22, which degrades PTEN leading to an increase in AKT activity. AKT phosphorylates FOXOs amongst other things. FOXOs normally acts as transcription factors leading to the transcription of genes that have been shown to promote cell cycle arrest and apoptosis such as CDK inhibitor, p27 and pro-apoptotic protein BIM. Phosphorylation of FOXOs by AKT, causes them to relocate from the nucleus to the cytoplasm where they are later degraded, thereby, allowing the cell cycle to progress. CD40 stimulation + IL-4 results in modest proliferation. CD40 stimulation + IL-21 results in more profound proliferation. The JAK kinase inhibitor, Ruxolitinib, has been shown to inhibit near to 100% CD154 + IL-21 induced proliferation (Pascutti et al., 2013). In my study, AKT inhibitors, MK-2206 and AZD5363 were shown to inhibit CD154 + IL-4 induced proliferation by 50%, and shown to decrease CD154 + IL-21 induced proliferation in 3/5 cases tested.

6.3 Expression of AKT and PI3K in CLL cells

Prior to undertaking this project, expression of isoforms of class I PI3K p110 subunits has not been well studied in CLL cells except that p110 δ has been reported to be abundantly expressed in these cells (Herman et al., 2010). During the time of my PhD study, I examined at the protein level the expression of PI3K p110 subunits in unstimulated CLL cells. I found that compared to normal B cells CLL cells have expressed higher levels of p110 δ and p110 γ , and decreased level of p110 β and similar level of p110 α . Experiments using purified CLL cells again showed that PI3K p110 γ was present in CLL cells and that the level of PI3K p110 γ was higher in CLL cells than normal B cells.

The effect of CD40 stimulation on expression of total AKT, phosphorylated AKT and PI3K p110 isoform was examined. CD40 stimulation appeared to maintain AKT activation as measured by p-AKT Ser473 and Thr308, but the total amount of AKT seemed to have decreased. Exactly why total AKT expression goes down upon CD40 stimulation is unclear. It may be possible that the total AKT antibody has a lower affinity for the hyperphosphorylated form of AKT. Most likely, it is because AKT upon phosphorylation at serine 473 is targeted for ubiquitination and rapid proteasomal degradation (Wu et al., 2011).

PTEN expression was confirmed to be decreased upon CD40 stimulation, inkeeping with the literature (Palacios et al., 2014, Palacios et al., 2015). CD40 stimulation decreased expression of PI3K p110 α whilst having no effect on the expression of PI3K p110 β in CLL cells. In contrast, the expression of both PI3K p110 δ and p110 γ isoforms was up-regulated in CD40-stimulated CLL cells (Figure 6.3). These results raise the question of what role of PI3K p110 δ and p110 γ play in CLL cells in response to CD40 stimulation.

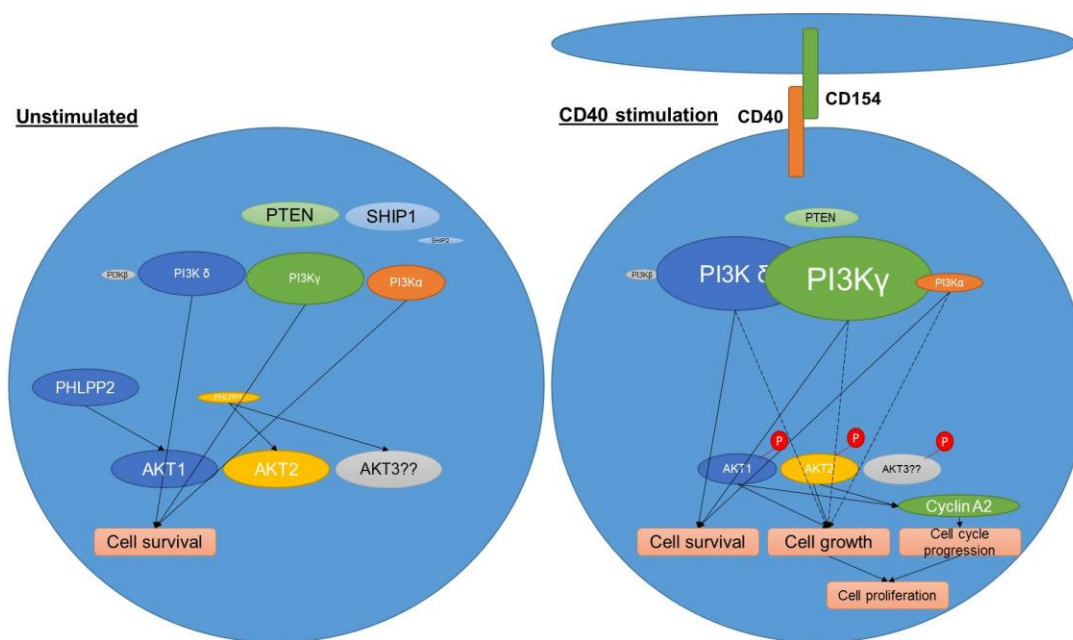


Figure 6.3: Expression of PI3K-AKT pathway components in unstimulated and CD40 –stimulated CLL cells.

This model is based on information from the literature and my own findings. In unstimulated cells, PTEN, SHIP1 and PHLPP2 are all present, PI3K δ and PI3K γ are abundantly present, PI3K α is less present, whilst PI3K β has very low expression (left hand side). CD40 stimulation leads to decreased PTEN, increased PI3K δ and PI3K γ , decreased total levels, but phosphorylated AKT, which results in cell growth, cell cycle progression and cell proliferation (right hand side).

Based on my own findings: Compared to normal B cells CLL cells have expressed higher levels of p110 δ and p110 γ , and decreased level of p110 β and similar level of p110 α . CD40 stimulation appeared to activate AKT as measured by p-AKT Ser473 and Thr308. CD40 stimulation decreased expression of PTEN and PI3K p110 α and increased expression of PI3K p110 δ and p110 γ .

Based on the literature: AKT1 and AKT2 are now known to be expressed in CLL and AKT1 was shown to be required for survival and chemoresistance of CLL cells, despite AKT2 being more highly expressed at both mRNA and proteins levels (Zhuang et al., 2010, Hofbauer et al., 2015). The expression of AKT3 in CLL cells has not been reported. PHLPP2 (PH domain leucine-rich repeat protein phosphatase) is present in CLL cells, but PHLPP1 was significantly reduced in primary CLL cells (Suljagic et al., 2010, O'Hayre et al., 2012). In another cell line, PHLPP1 is known only to interact with AKT2 and AKT3, in this case, knockdown of PHLPP1 increased hydrophobic motif phosphorylation of only AKT2 and AKT3 (Mendoza and Blenis, 2007). Recent investigations studying PHLPP1 in CLL detected PTEN protein expression in all CLL samples studied (O'Hayre et al., 2012). Most recently, PTEN mRNA and protein were found to be down regulated in CLL cells co-cultured with CD40 +IL-4 (Palacios et al., 2014). Finally, whilst SHIP2 protein was undetectable, SHIP1 protein was detected in primary CLL cells (Gabelloni et al., 2007).

6.3.1 The role of PI3K p110 δ in CLL

Survival, adhesion and migration are some of the functions of p110 δ in CLL cells were recently discovered using the p110 δ isoform selective inhibitor, idelalisib ([Section 1.4.1.4](#)). PI3K p110 δ has been shown to mediate the survival signals provided by soluble CD40L stimulation, BAFF ligation, TNF α and direct contact with stromal cells or a fibronectin support (Herman et al., 2010). p110 δ also mediates BCR-stimulated and NLC induced secretion of CCL3, CCL4 and CXCL13 by CLL cells (Hoellenriegel et al., 2011, Balakrishnan et al., 2015).

Co-culturing CLL cells with NLCs results in increased CCL2, CCL7, IL-6, sCD154, CCL22, TNF α and CCL17 secretion and the addition of idelalisib reduced the secretion of all of these chemokines and cytokines (Hoellenriegel et al., 2011).

The migration of CLL cells towards CXCL12 and CXCL13 is also dependent upon p110 δ (Hoellenriegel et al., 2011, Balakrishnan et al., 2015). Recently it was shown that p110 δ controls expression of CXCR4 and CD49d (both molecules are important in CLL migration) (Pepper et al., 2015). p110 δ is also required for integrin-mediated adhesion of CLL cells to endothelial and marrow stromal cells and enhanced survival of the leukaemic cells as its inhibition by idelalisib prevented adhesion of CLL cells to both stromal cells and obliterated stromal cell-mediated protection (Fiorcari et al., 2013). Further, BCR signalling suppresses S1PR1 expression via a mechanism involving PI3K p110 δ , thereby preventing CLL cells from egressing the lymph node environment (Till et al., 2015).

6.3.2 The role of PI3K p110 γ in CLL

The finding that PI3K p110 γ was expressed by CLL cells was novel. Although PI3K p110 isoforms are highly expressed in leukocytes, deletion of p110 γ did not cause major defects in the development or function of B cells in mice (Sasaki et al., 2000). At the mRNA level, PI3K p110 γ expressed was found to be expressed at significantly lower levels in normal B cells than in dendritic cells, NK cells and myeloid cells and below the average expression from a panel of 79 human tissues (Figure 6.4) (Su et al., 2004). In contrast a more recent study did show that PI3K p110 γ was expressed in B cells at early developmental stages (Beer-Hammer et al., 2010). Beer-Hammer and colleagues showed that p110 γ and p110 δ have overlapping

roles in the development, survival and proliferation in B cells (Beer-Hammer et al., 2010). Mice with p110 γ /p110 δ double deficiency had less mature splenic B cells and these splenocytes were less responsive to LPS or CD40 stimulation (Beer-Hammer et al., 2010).

PI3K p110 γ is required for cellular migration by some immune cells (Okkenhaug, 2013). PI3K p110 γ is vital for T cell development and activation (Sasaki et al., 2000) and important in CXCL-12-mediated T cell migration (Sawyer et al., 2003, Martin et al., 2008, Reif et al., 2004) and essential for efficient induction of CXCR3 on activated T cells (Barbi et al., 2008). PI3K p110 γ is involved in neutrophil migration (Sasaki et al., 2000, Randis et al., 2008) and is activated by receptor tyrosine kinases and TLRs in myeloid cells (Schmid et al., 2011). Gene knockout study in mice showed that PI3K p110 γ is also important for dendritic cell migration (Del Prete et al., 2004). What is the physiological function of PI3K p110 γ in CLL cells? p110 γ signals downstream of G-protein coupled receptors (GPCRs) (Stoyanov et al., 1995, Stephens et al., 1997, Stephens et al., 1994). There are over 100 GPCRs expressed on CLL cells (Katakia, 2012), among them are chemokine receptors: CXCR4, CCR7 and CXCR3 (Balkwill, 2012). CXCR4 – CXCL12 signalling is critical for adhesion of CLL cell to BMSC (Burger et al., 1999, Burger, 2011) and NLCs (Burger et al., 2000, Nishio et al., 2005). CCR7 is involved in the migration of B cells into the lymph nodes (Herman et al., 2011a, Hendriks et al., 2014). CXCR3 mediates chemotaxis of CLL cells (Trentin et al., 1999). It is therefore not unreasonable to speculate that PI3K p110 γ may be involved in the migration of CLL cells. Targeting PI3K p110 γ in CLL may therefore be a sensible strategy to dislodge leukaemic cells from lymphoid tissues, especially in patients who have developed lymphadenopathy.

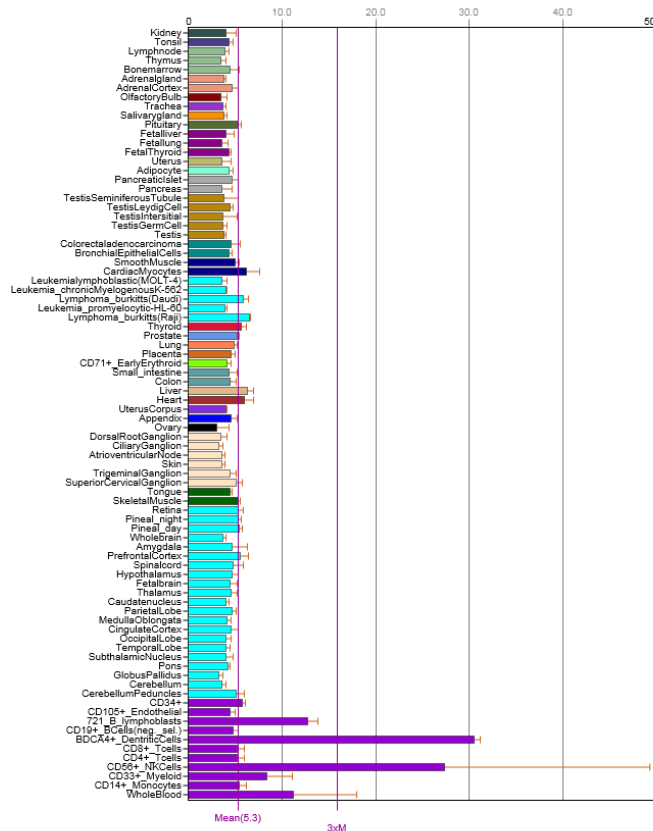


Figure 6.4: PI3K p110 γ gene: PIK3CG expression in Gene Atlas profile (GeneAtlas U133A, grma).

Image from: <http://biogps.org/#goto=genereport&id=5294>

The tissue-specific pattern of mRNA expression can indicate important clues about gene function. High-density oligonucleotide arrays offer the opportunity to examine patterns of gene expression on a genome scale. Toward this end, we have designed custom arrays that interrogate the expression of the vast majority of protein-encoding human and mouse genes and have used them to profile a panel of 79 human and 61 mouse tissues. The resulting data set provides the expression patterns for thousands of predicted genes, as well as known and poorly characterized genes, from mice and humans. We have explored this data set for global trends in gene expression, evaluated commonly used lines of evidence in gene prediction methodologies, and investigated patterns indicative of chromosomal organization of transcription. We describe hundreds of regions of correlated transcription and show that some are subject to both tissue and parental allele-specific expression, suggesting a link between spatial expression and imprinting. A gene atlas of the mouse and human protein-encoding transcriptomes (Su et al., 2004)

Indeed, preclinical studies with p110 γ inhibitors in primary CLL cells have provided some proof of principle. For example, Balakrishnan and colleagues showed that dual PI3K δ/γ inhibitor duvelisib inhibited CLL-cell chemotaxis towards CXCL12 and migration beneath stromal cells (Balakrishnan et al., 2015).

6.3.3 PI3K regulatory subunits in CLL

Knocking out p110 δ isoform in mice also affects the expression at the protein level of p85 α , p55 α and p50 α (Clayton et al., 2002). Furthermore, B-cell development and proliferation was impaired in the absence of PI3K regulatory subunit p85 α (Fruman

et al., 1999b, Fruman et al., 1999a), indicating that the expression of PI3K regulatory subunits also affects the function of PI3K kinases. In future it might be relevant to study the expression of the regulatory subunits of PI3K in CLL cells and determine whether inhibiting PI3K p110 isoforms with isoform-selective inhibitors such as idelalisib will change the expression levels of the regulatory subunits.

6.4 AKT-independent mechanisms of PI3K action

The effect of PI3K inhibitors on survival and proliferation of CD40-stimulated CLL cells was more profound than that of AKT inhibitors, AZD5363 and MK-2206, suggesting PI3K may exert its effects in CLL partly through AKT-independent mechanisms. Currently, there is limited information in the literature regarding such mechanisms. Nevertheless, MAPK signalling has been suggested as one such AKT-independent pro-survival pathway that is activated by PI3K (Rahmani et al., 2003). The BTK signalling pathway can be activated downstream of PI3K and is another AKT-independent signalling pathway (Mohamed et al., 2009). The SGK3 signalling pathway has not been investigated in CLL, but is an AKT-independent signalling pathway in other cancers, and thus could be investigated in CLL (Bruhn et al., 2010, Bruhn et al., 2013, Park et al., 1999, Kobayashi et al., 1999).

6.5 Therapeutic potential of AKT inhibition in CLL

The data presented in my thesis suggest that AKT inhibition by the inhibitor AZD5363 or MK-2206 would not be very effective as a cytoreductive approach in therapy if the inhibitors were used as monotherapy since it resulted in little or no killing of CLL cells under co-culture conditions that mimic the lymph node microenvironment. However, the AKT inhibitors AZD5363 or MK-2206 in combination with cytotoxic agents such as bendamustine may be useful in killing tissue resident CLL cells. Furthermore, AKT inhibition by either AZD5363 or MK-2206 can inhibit CLL-cell proliferation in some but not all CLL cases. Further investigation is clearly required to understand which patients would benefit most from the therapeutic strategy of AKT inhibition.

AKT inhibition in combinations with other novel agents may also have therapeutic potential. For example, PIM kinases are important in mediating CXCR4-induced migration of CLL cells in the microenvironment and PIM kinase inhibitors have been shown to induce apoptosis in CLL (Chen et al., 2009, Decker et al., 2014). It is

possible that combining AKT and PIM kinase inhibitors in CLL cells may yield synergism and could be further investigated. AZD5363 has been shown to synergise with a PIM inhibitor in AML cell lines (Meja et al., 2014).

A recent pre-clinical *in vitro* study investigated the effects of triple AKT inhibition by GSK690693, MK-2206 and Perifosine in T-ALL cell lines and reported significant synergistic and cytotoxic effects (Cani et al., 2015). An approach like this may also have therapeutic potential in CLL, however this may also give toxicity concerns.

6.6 Reflections on working with primary CLL cells

6.6.1 Proliferation assays

Much of my work was based on the use of an *in-vitro* model of CD40-mediated proliferation of CLL cells. There is an incomplete knowledge about CD40 signalling in CLL cells and further studies to improve our understanding of CD40 signalling in CLL cells are desirable. Furthermore, there is conflicting literature regarding the best models to use for studying CLL-cell proliferation *in vitro*. Naive and memory mature B cells undergo differentiation into Ab-secreting cells by stimulation with CD40L and cytokines (Neron et al., 2011). Thus some have suggested that CD40 stimulation leads CLL cells to differentiate into Ab-secreting cells. However, it was recently shown that phorbol myristate acetate, but not CD40 stimulation, induce the differentiation of CLL cells into antibody-secreting cells (Ghamlouch et al., 2014). Recent studies showed that CD40-stimulated cells had a phenotype and morphology similar to those of activated B cells and, from a gene expression profiling perspective, they closely resembled CLL cells residing in the lymph node and bone marrow. CLL cells co-cultured with CD154-expressing fibroblasts alone only caused some *IGHV*-unmutated CLL samples to proliferate, but not mutated-CLL cases (Tromp et al., 2010). The addition of cytokines to CD40-stimulation induced CLL cells from most patients to proliferate, irrespective of their *IGHV* mutational status (Patten et al., 2012). These cytokines include IL-4 (Buske et al., 1997, Frenquelli et al., 2010, Palacios et al., 2014), IL-4 + IL-21 (Ahearne et al., 2013) or IL-21 (Pascutti et al., 2013). Alternatively, CLL cells co-cultured with stromal cells in the presence of soluble CD40L + IL-2 + IL-10 can also be induced to proliferate (Plander et al., 2009).

Throughout my PhD project, I added IL-4 or IL-21 to CD40 stimulation by co-culturing CLL cells with CD154-expressing fibroblasts to induce proliferation of CLL cells.

My earlier experiments showed that CD40 + IL-4 stimulation resulted in CLL-cell proliferation after seven days, which does not seem dependent on the *IGHV* mutational status of the CLL cells. IL-4 is produced by T cells and signals via receptor mediated JAK1 and JAK3 phosphorylation, leading to the activation of transcription factor STAT6 (Nelms et al., 1999), and induced increased expression of MCL1 and BCL-XL, which prolongs CLL-cell survival *in vitro*. IL-4, whilst activating the JAK3-STAT6 pathway, also activated ERK-RSK-S6 in CLL cells, but not the AKT pathway (Steele et al., 2010).

Later, IL-21 was shown to be involved in CLL-cell proliferation (Pascutti et al., 2013, Ahearne et al., 2013). In normal B cells, depending on the interplay between co-stimulatory signals and on the developmental stage of a B cell, IL-21 has been shown to induce proliferation, differentiation into antibody producing plasma cells, or apoptosis (Ettinger et al., 2005, Jin et al., 2004). In CLL cells, initial studies showed that IL-21 failed to induce proliferation but promoted apoptosis (de Toter et al., 2008, de Toter et al., 2006, Gowda et al., 2008).

IL-21 induces JAK-1 and JAK-3 autophosphorylation and tyrosine phosphorylation of STAT-1, STAT-3 and STAT-5 upon CD40 stimulation in CLL cells (de Toter et al., 2006, de Toter et al., 2008). Whilst IL-21 alone induced apoptosis in 6/20 cases in resting CLL cells, a more potent effect of IL-21 was seen upon CD40 stimulation. IL-21 induced apoptosis of CD40-activated CLL B cells in a time (taking between 48 -72 hours) and dose-dependent manner (de Toter et al., 2006). This more potent increase in cell death may be because IL-21R was shown to be upregulated by CD40 triggering (de Toter et al., 2006). IL-21 was shown to induce apoptosis in CD40-activated cells via activation of caspase-8 and caspase-3, cleavage of Bid to its active form t-Bid, and cleavage of PARP and p27Kip-1 (de Toter et al., 2006). CD40-stimulated CLL cells treated with IL-21 appears to induce apoptosis via a delayed, extrinsic pathway of apoptosis (de Toter et al., 2006, Gowda et al., 2008). However, IL-21R has no death domain and therefore it cannot directly trigger apoptosis via Fas-associated death domain (FADD)/caspase-8 (de Toter et al., 2006). Treatment

of resting CLL cells with IL-21 alone was shown to induce BIM up-regulation (an apoptotic protein), which caused apoptosis in CLL cells (Gowda et al., 2008).

However, Pascutti and colleagues showed that CD40 stimulation in the presence of IL-21 was a potent inducer of CLL-cell proliferation *in vitro*, and IL-21 was physiologically relevant as it was detected by immunohistochemistry in the lymphnode biopsy samples from patients with CLL (Pascutti et al., 2013). Later, it was shown that IL-21 also enhanced CD40 + IL-4-induced proliferation of CLL cells (Ahearne et al., 2013). I showed that CD40 + IL-21 is more potent than CD40 + IL-4 in inducing CLL-cell proliferation. It is worth noting that the earlier papers that reported apoptosis was induced by IL-21 in combination with CD40 stimulation used higher concentrations of IL-21 (20-200 ng/ml) (Gowda et al., 2008, de Toter et al., 2006, de Toter et al., 2008) rather than the 15 ng/ml used in this project or 25 ng/ml used by Eric Eldering's group (Pascutti et al., 2013). In my hands there does appear very small, but not substantial amount of cell death (as measured by PI) in CLL cell stimulated with CD40L +IL-21 compared to those cultured on control fibroblasts (Figure 4.15) (though I did not control for CD40L without IL-21 stimulation).

Whilst, CLL cells normally express IgD and IgM. There is evidence to suggest that immunoglobulin class switch recombination (CSR) from IgM to IgG may occur upon treatment with CD40L and IL-4 (Cerutti et al., 2002) and that the CLL cells may even express IgE in response to CD40L and IL-4 (Malisan et al., 1996). It has also been shown that stimulation of CLL cells CD40L and IL-10 induced CLL cells to secrete IgG and IgA (Malisan et al., 1996). Immunoglobulin class switch recombination is reviewed further by Lanasa and Weinberg (Lanasa and Weinberg, 2011). Whether this system truly represents how CLL proliferates *in vivo* to produce more IgM+ CLL cells, require much further study.

6.6.2 Crosstalk between CLL cells and stromal cells

As stated earlier, I did not study signalling events in CLL cells or CD154-expressing fibroblasts after co-culture. Ringhausen and colleagues showed that the culture of CLL cells upon murine stromal cells (EL08-1D2) changed expression of proteins not only in CLL cells but also in the stromal cells (Lutzny et al., 2013). Culturing CLL cells on murine stromal cells (EL08-1D2) induced (IL-1 α) and IL-15 expression in stromal cells. These cytokines in turn protected CLL cells from apoptosis. This study

uncovered a signalling mechanism responsible for the cross talk between cancer cells and stromal cells, thus providing evidence that CLL microenvironment can be actively modulated by CLL cells (Seton-Rogers, 2013). It may be interesting to study if such cross talk is occurring in our model.

6.6.3 Application of gene transfer techniques in primary CLL cells

Attempts at knocking down specific AKT isoforms using a lentiviral delivery of shRNA approach were tried but eventually abandoned due to lack of progress. Primary CLL cells are notoriously difficult to transfect with most gene transfer techniques including electroporation, or permeabilisation with chemical reagents such as lipofectamine, probably due to their non-proliferating nature. Seiffert and colleagues showed that using the Amaxa nucleofection technology they were able to transfect primary CLL cells with efficiencies varying between 50 -70% (Seiffert et al., 2007). However, viability of transfected CLL cells 24 hours after nucleofection was between 20-60%. Furthermore, it has also been reported that gene transfer by adenoviral vector transduction in CLL cells was inefficient because CLL cells lack the adenoviral receptor (Frecha et al., 2009). However, by stimulating CLL cells through the BCR prior to adenoviral infection, Frecha and colleagues managed to increase infection efficiency to 80% (Frecha et al., 2009). Most encouragingly, Pearce and colleagues were able to perform lentiviral infection method to deliver gene transfer in primary CLL cells (Pearce et al., 2010). In order to achieve high viability of CLL cells post transfection and stable expression of siRNA, I attempted to knock down AKT1 and AKT2 using shRNA delivered by lentivirus infection. Unfortunately these attempts were unsuccessful. Since there is some evidence that stimulated CLL cells might be easier to be infected I repeated these experiments using CLL cells that have been co-cultured with CD154-expressing fibroblasts for 24 hours prior to lentiviral infection (Frecha et al., 2009). Again, I could not make any improvement using this method on transfection efficiency. An approach that consistently yields high infection efficiency coupled with low induction of apoptosis is vital in future CLL research. Other technologies such as use of silicon nanowires that Wang and colleagues have recently described could be a useful new transfection method (Wang et al., 2014).

6.6.4 Detecting AKT activation

If I were to perform further studies regarding the AKT phosphorylation in CLL cells, I would also like to use another methodology, e.g. to measure AKT phosphorylation by flow cytometry in addition to Western blotting. To perform Western blotting, one needs a large amount of cells to begin with. In my case, I used 4×10^6 cells and then lysed in 100 μ l RIPA buffer. Usually there are always some CLL cells firmly adhering to the monolayers of fibroblasts after co-culture, making it impossible to harvest all CLL cells initially seeded. Typically, the recovery rate of co-cultured CLL cells at the end of incubation is about 50%, thus requiring the minimum number of 10×10^6 CLL cells per treatment if Western blotting analysis is to be used. In contrast, for flow cytometry analysis one usually needs less than 1×10^6 cells per treatment, thus significantly reducing the amount of CLL cells needed. p-AKT in CLL has now been examined in flow cytometry by several groups, either at serine 473 (Balakrishnan et al., 2015) or threonine 308 (Palacios et al., 2014).

6.7 Summary

In summary, I have used a CD154-expressing fibroblast co-culture system to model protective lymph node environments where CLL cells interact with T cells through the CD40-CD154 interaction, resulting in enhanced survival and proliferation. I hypothesised that PI3K-dependent activation of AKT mediates both the cytoprotective and proliferative effects of CD40 in the presence of IL-4/IL-21 in primary CLL cells. To test this hypothesis, I set out to address the following research questions and here summarise their respective main findings obtained through my PhD studies:

1. To what extent does AKT contribute to the survival of CLL cells cultured under conditions that mimic the lymph node microenvironment?

I showed that AKT activation was maintained in CD40 stimulated CLL cells and that AKT is important in mediating CD40-stimulated survival of CLL cells, as inhibiting AKT by AZD5363 decreased baseline viability and also increased bendamustine-induced killing.

2. To what extent does AKT contribute to the proliferation of CLL cells induced by CD40L + IL-4/IL-21?

I showed that AKT was required for growth of CLL and normal B cells stimulated with CD154 + IL-4 or CD154 + IL-21. I have also shown that CLL cells require AKT for proliferation induced by CD154 + IL-4, as inhibition of AKT by AZD5363 significantly reduced such proliferation. However, normal B cells did not require AKT in CD154 + IL-4-stimulated proliferation, therefore indicating a selective requirement for AKT in such proliferation from CLL cells. Furthermore, I have showed that AKT is required for CD40 + IL-21-induced proliferation in CLL cells from some (3/5) but not all patient samples. Again, normal B cells did not require AKT in CD154 + IL-21 stimulated proliferation. Inhibition of AKT by AZD5363 inhibited induction of cyclin A2 and CDK1 in CD40 + IL-21-stimulated CLL cells. Further studies would be required to understand why the inhibition of AKT inhibits CD154 + IL-21-stimulated proliferation in CLL cells from some but not other cases.

3. To what extent does PI3K regulate activation of AKT in CD40-stimulated CLL cells?

I have shown that CD40 stimulation decreased expression of PI3K p110 α whilst having no effect on the expression of PI3K p110 β in CLL cells. In contrast, the expression of both PI3K p110 δ and p110 γ isoforms were upregulated in CD40 stimulated CLL cells. I also showed that the PI3K p110 δ isoform is mainly responsible for the activation of AKT in CLL cells in response to CD40 stimulation, but the p110 γ isoform may also play a role. Further studies on the function of PI3K p110 γ in CLL cells are required to establish its role in CLL.

6.8 Conclusion

In conclusion, I have demonstrated a role for AKT in CLL-cell survival and cell growth (increase in cell size) upon CD40 stimulation. I have also shown that AKT is involved in mediating CD40-stimulation-induced resistance to bendamustine. However, the requirement of AKT for CD40 stimulation induced CLL-cell proliferation is dependent on the cytokines used and varies between patients. I showed for the first time that both PI3K p110 δ and p110 γ isoforms were upregulated in CD40-stimulated CLL cells. Moreover, my data showed that PI3K p110 δ isoform is mainly responsible for the activation of AKT in CLL cells in response to CD40

stimulation, and that p110 γ isoform may also play a role. Therefore my studies have demonstrated that the PI3K-AKT pathway plays an important role in CLL-cell survival, growth, drug resistance and proliferation in response to CD40 stimulation, which is highly relevant to the lymph node microenvironment.

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Appendix

1. Protocols

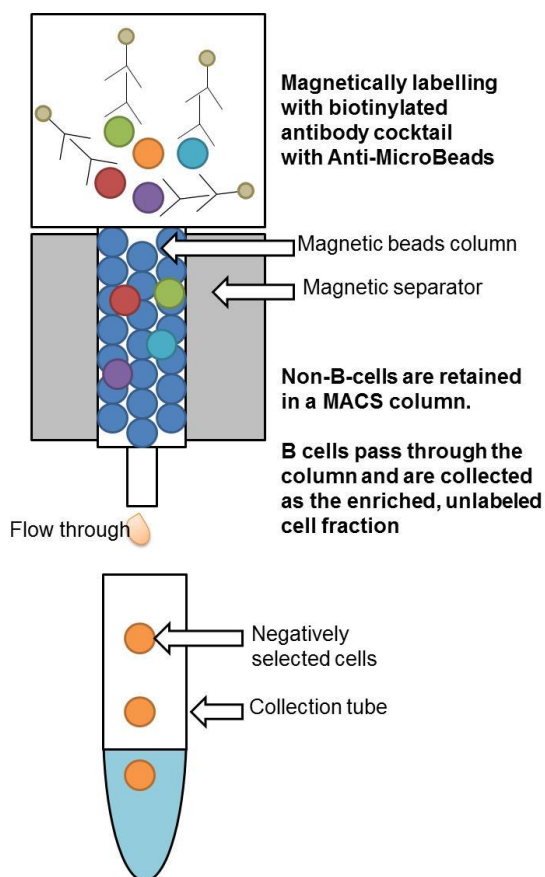
1.1 Purification of CLL B cells.

B cells were isolated from CLL PBMCs using the B-CLL Cell Isolation Kit, human (Cat. no.130-103-466, Miltenyi Biotec Ltd). Practically, a buffer containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA was made, and allowed to degas overnight prior to use. The buffer was kept cold (4°C) and CLL PBMC cells were resuspended in 40 µl of buffer per 10⁷ total cells. 10 µl of Biotin-Antibody Cocktail per 10⁷ total cells was added (Containing biotin-conjugated antibodies against non CLL B cells: CD2, CD3, CD4, CD14, CD15, CD16, CD56, CD61, CD235a, FcεRI and CD34). Cells were mixed well with the antibody cocktail and incubated for 10 minutes at 2–8 °C. A further 30 µl of buffer was added per 10⁷ total cells and 20 µl of Anti-Biotin MicroBeads per 10⁷ total cells. This mixture was well mixed and then incubated for 15 minutes at 2–8 °C. Cells were washed by adding 1–2 mL of buffer per 10⁷ cells and centrifuging at 300g for 10 minutes. The supernatant was decanted and the cell pellet was resuspended in 500 µl of buffer per 10⁸ cells. A LS-MACS Separation column (130-042-401) was placed in the magnetic field, the column was prepared by rinsing with 3 mL of buffer. The cell suspension was applied via a MACS 30 µm pre-separation filter (130-041-407) onto the column. The column was washed three times with 3 mL of buffer. The unlabelled cells were collected combined with the flow-through. B cell purity was assessed by CD19 positivity, using CD19-PE antibody and control-PE antibodies on FL-2 on the BD FACSCalibur™.

1.2 Normal B cells isolated from normal healthy donors

Normal B cells were isolated from the PBMCs obtained from healthy donors using the B Cell Isolation Kit II (Cat. no. 130–091–151, Miltenyi Biotec Ltd). Practically, a buffer containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA was made, and allowed to degas overnight prior to use. The buffer was kept cold (4°C) and cells were resuspended in 40 µl of buffer per 10⁷ total cells. 10 µl of Biotin-Antibody Cocktail per 10⁷ total cells was added (Containing biotin-conjugated antibodies against non B cells: CD2, CD14, CD16,

CD36, CD43, and CD235a (glycophorin A)). Cells were mixed well with the antibody cocktail and incubated for 10 minutes at 2–8 °C. A further 30 µL of buffer was added per 10^7 total cells and 20 µl of Anti-Biotin MicroBeads per 10^7 total cells. This mixture was well mixed and then incubated for 15 minutes at 2–8 °C. Cells were washed by adding 1–2 mL of buffer per 10^7 cells and centrifuging at 300g for 10 minutes. The supernatant was decanted and the cell pellet was resuspended in 500 µl of buffer per 10^8 cells. A LS-MACS Separation column (130-042-401) was placed in the magnetic field, the column was prepared by rinsing with 3 mL of buffer. The cell suspension was applied via a MACS 30 µm pre-separation filter (130-041-407) onto the column. The column was washed three times with 3 mL of buffer. The unlabelled cells were collected combined with the flow-through. B cell purity was assessed by CD19 positivity, using CD19-PE antibody and control-PE antibodies on FL-2 on the BD FACSCalibur™.



Appendix Figure 1: Isolation of normal B cells using the B Cell Isolation Kit II.

Non-B cells, such as T cells, NK cells, dendritic cells, monocytes, granulocytes, and erythroid cells (depicted by the red, purple, blue and green circles), are indirectly magnetically labelled by using a cocktail of biotin conjugated antibodies against CD2, CD14, CD16, CD36, CD43, CD235a Glycophorin A, and Anti-Biotin MicroBeads. These are trapped in the magnetic column, whereas the unlabelled B cells (depicted by the orange circles) pass through and are eluted from the column.

1.3 Co-culture of CLL cells with CD154-expressing fibroblasts

CLL cells were co-cultured with the CD154-expressing or control fibroblasts at a ratio of 10 to 1 for 24h or 48h in the presence or absence of the above drugs before harvesting. Practically, fibroblasts were gamma irradiated at $6-8 \times 10^5$ cells/ml for 20 minutes at 30 Gy to inhibit cell growth, and were then diluted 50:50 with the fresh medium and finally seeded at $3-4 \times 10^5$ cells in a 24-well plate (or for other plate sizes, see Appendix Table 1). Cells were left to adhere and recover overnight. 3×10^6 CLL cells (in one ml of RPMI medium) were added to the monolayer with additional drugs or cytokines.

Appendix Table 1: Co-culture of CLL cells with CD154 expressing cells

	Fibroblasts			CLL		
	Cell conc. (cells/ml)	Volume (ml)	Total (Cells)	Cell conc. (cells/ml)	Volume (ml)	Total (Cells)
24-well	$3-4 \times 10^5$	1	3×10^5	3×10^6	1	3×10^6
48-well	3×10^5	0.5	1.5×10^5	3×10^6	0.5	1.5×10^6
96-well	5×10^5	0.1	0.5×10^5 5×10^4	5×10^6	0.1	0.5×10^6 5×10^5

1.4. CFSE labelling of CLL cells

CLL cells in RPMI at 3×10^6 cells/ml were labelled with CFSE by the addition of 1:1000 dilution of 0.5 mM CFSE (cat. no. C34554, Molecular Probes via Life Technologies Ltd, Paisley, UK) to make a final concentration of 0.5 μ M CFSE and incubated at 37°C for 10 minutes. CLL cells were then spun at 550g for 5 min, the supernatant discarded, and cell resuspended in fresh complete RPMI medium and incubated for a further 20 minutes at 37°C. Labelled CLL cells were then washed (spun at 550g for 5min, the supernatant discarded, and cell resuspended) twice and finally re-suspended in complete RPMI medium.

1.5 shRNA lentiviral production

The lentivirus packaging plasmid (psPAX2) and envelope glycoprotein plasmid (pMD2.G) were a gift from Didier Trono (Addgene plasmids # 12260 and 12259

respectively). Plasmid pLKO.1-puro-CMV-TurboGFP (Sigma Aldrich, UK; catalogue number SHC003) was used for measuring transfection efficiency and optimizing shRNA delivery. This consists of the lentiviral backbone vector, pLKO.1-puro, containing a gene encoding TurboGFP driven by the CMV promoter. A plasmid expressing a non-targeting shRNA molecule was used as a negative control (Sigma Aldrich UK; catalogue number SHC002),

1.5.1 Plasmid DNA midi-prep.

Plasmids were purified from *E.coli* grown in 100ml terrific broth (Sigma Aldrich, UK) using the PureYield™ Plasmid Midiprep kit (Promega, UK) as per the manufacturers protocol. Briefly, bacteria were pelleted at 5,000 g for 10 minutes, the supernatant discarded and the bacterial pellet re-suspended in 6 ml of Resuspension Solution. Bacteria were lysed by the addition of 6ml Cell Lysis Solution, followed by inversion to completely mix the solution. The lysate was incubated for 3 minutes at room temperature, after which 10ml Neutralization Solution was added and mixed well. This lysate was then centrifuged at 15,000g for 15 minutes at room temperature to pellet insoluble material. The cleared supernatant was passed through both the blue PureYield™ Clearing Column and the white PureYield™ Binding Column by centrifugation. The PureYield™ Binding Column was rinsed with Wash Solution and allowed to dry. Finally the plasmid DNA was eluted by adding 600 µl of Nuclease-Free Water to the DNA binding membrane in the binding column and centrifuging.

1.5.2 Lentivirus production

Approximately 18 hours prior to transfection, 1×10^7 293T cells in 25ml DMEM/10% FBS were seeded in T175 flasks. At the time of transfection, the cells were approximately 70% confluent. Immediately before transfection chloroquine (Sigma Aldrich, UK) was added to each flask to a final concentration of 25 µM. For each flask, the following transfection mix was prepared in a 30ml universal: 14 µg packaging plasmid psPAX2, 7 µg envelope glycoprotein (VSV-G) plasmid pMD2G, 21 µg transfer plasmid (which contains either the shRNA of interest) or pLKO.1-puro-CMV-TurboGFP, 389 µl 2M CaCl plus H₂O to final volume of 3ml. To this was added 3ml of 2X HBS and the pipette was used to slowly bubble air through the solution for 1 minute. The transfection mixture was then slowly

added to the cells, and the flasks returned to the incubator. After 24 hours, media was replaced with 25 ml of fresh DMEM/10% FBS per flask and the cells incubated for a further 24 hours. Media was replaced every 24 hours (for three days) and the harvested media was kept at 4°C. Following the final collection, the virus-containing media was centrifuged at 500g for 10 min to remove cellular debris then filtered through a 0.45 µm filter.

1.5.3 Lentivirus concentration

Lentiviral particles were concentrated by ultracentrifugation. Briefly, the media was transferred into a suitable centrifuge tube & centrifuged at 30000g for 2 h at 15°C. The supernatant was carefully decanted, and the tube was further spun at 500g for 5 minutes. Residual liquid was removed and the pellet resuspended in 500 µl PBS. The tubes were stored at 4°C for 2 h and vortexed every 15 min to resuspend the viral pellet. Finally the tubes were centrifuged at 500g for 5 min to pull down all virus-containing liquid. The liquid was aliquotted into cryotubes and stored at -80°C.

1.5.4 Titration of lentiviral particles by qRT-PCR of viral gag gene

In order to remove any residual plasmid DNA from the transfection, contaminating genomic DNA from the producer cells or any viral RNA that is not encapsulated, 1 µl of the concentrated lentiviral solution was incubated at 37°C for 15 minutes in a final volume of 100 µl with 2 µl DNase, 10 µl DNase buffer and 50 pg RNaseA.

Viral RNA was then purified using Qiagen viral RNA mini kit following the manufacturers' protocol and cDNA generated using the viral RNA in a reverse transcription reaction using the following primer, which binds to the viral *gag* gene (5'-GCTGCTTGCTGTGCTTTTTTCTTAC-3').

The number of viral genomes was then determined by qPCR using 1 µl of the cDNA in a final volume of 25 µl. Standard curves were generated from serial dilutions of the psPAX2 plasmid (which contains the *gag* gene). containing $1 \times 10^3 - 1 \times 10^8$ plasmid copies/µl. All qPCR reactions were carried out in triplicate. The sequences of the primers targeting the *gag* gene are as follows: (For 5'-AAGGCTGTAGACAAATACT GGGAC-3'; Rev 5'-GCACACAATAGAGGGTTGCTACTG)

The exact number of viral particles in the sample was calculated using a back-calculation as follows: Assume qPCR gives a value of 5×10^4 copies/ μl . The PCR was carried out using 1 μl of cDNA out of a total of 25 μl . This cDNA was made using 10 μl of the RNA, but there was 60 μl total. The RNA was prepared using 1 μl lentiviral sample. Thus, the actual viral titre is: $(5 \times 10^4) \times (25) \times \left(\frac{60}{10}\right) = 7.5 \times 10^6$ LV particles/ μl . The total number of virions would be then be 3.75×10^9 (assuming the concentrated lentiviral particles were resuspended in 500 μl).

1.6 Protein concentration determination

Protein concentration was determined using the Bio-Rad DCTM (detergent compatible) protein assay kit (cat. no. 500-0116, Bio-Rad laboratories, Hertfordshire, UK). Briefly, 5 μl of sample or 5 μl of standard curve were plated out in duplicate in a 96-well flat-bottomed plate, 25 μl of working reagent A' (1ml reagent A + 20 μl reagent S) was added to each well, followed by 200 μl of reagent B. The reaction was left for 15 minutes and then read on a plate reader at 630 nm.

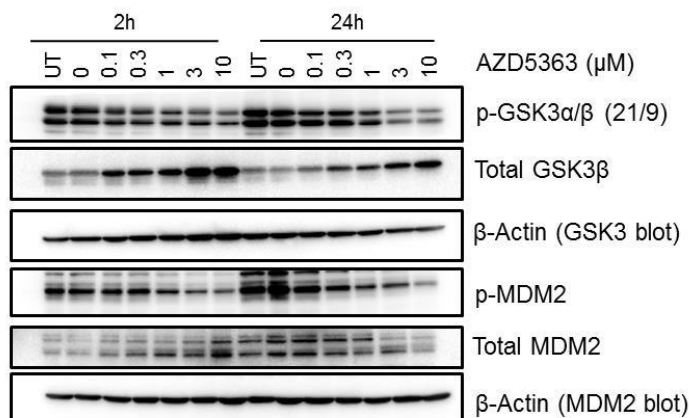
2. Chapter 3 Supplementary: The role of AKT in CLL-cell survival

2.1 AZD5363 inhibits AKT activity in Jurkat cells

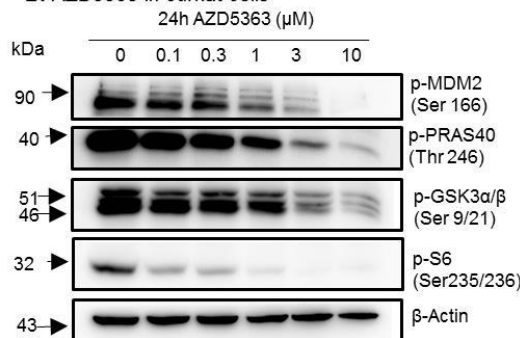
First, I sought to confirm the biological activity of AZD5363 using a human leukaemic cell line Jurkat cells as previously reported (Davies et al., 2012). The inhibitory activity of AZD5363 on AKT was measured by the loss of phosphorylation of several well-characterised AKT substrates using Western blotting analysis. I incubated Jurkat cells with a range of concentrations of AZD5363 for both 2 hours and 24 hours, and observed a concentration-dependent loss of phosphorylated GSK3 α/β and MDM2 (Appendix Figure 2A). There also appeared to be a concentration-dependent increase in total GSK3 β following treatment of AZD5363. Because total GSK3 β was re-probed on the same blots that have previously been probed for p-GSK3 α/β and these two antibodies were raised against the same epitope of the GSK3, I suspected that the first antibody used to probe p-GSK3 α/β may remain on the blots and interfere with the binding of the second antibody which was used to probe for total GSK3 β . Therefore in subsequent experiments, I used a different antibody which is specific to total GSK3 α/β . In addition, I probed for total GSK3 α/β using separate blots that have not been previously probed for p-GSK3 α/β . As a result, I consistently found that the

expression of total GSK3 α/β was not affected by the addition of AZD5363 (Appendix Figure 2C). I also examined phosphorylation status of some other AKT substrates, such as PRAS40 and the downstream substrate S6 in Jurkat cells incubated with AZD5363 for 24 h. As expected, incubation of Jurkat cells with AZD5363 resulted in a concentration-dependent loss of phosphorylation of PRAS40 and S6 (Appendix Figure 2B). Densitometric analysis of signals corresponding to respective levels of p-GSK3 α/β , p-PRAS40, p-MDM2 and p-S6 indicated that AZD5363 inhibited AKT activity with an estimated IC₅₀ between 0.1 and 0.3 μ M in Jurkat cells (Appendix Figure 2C), a result consistent with the previous findings (Davies et al., 2012). AZD5363 did not seem to induce any loss in viability after 24 hours as measured by trypan blue exclusion (Appendix Figure 2D). However it did appear to inhibit proliferation of the Jurkat cells, as addition of AZD5363 led to a consistent decrease in total number of cells (Appendix Figure 2E). In fact, 10 μ M AZD5363 resulted in almost 50% reduction in the number of viable cells when compared to that of untreated cells (Appendix Figure 2E). This indicated that the mode of action of this drug was mainly anti-proliferative rather than cytotoxic, which was consistent with the observation previously reported (Davies et al., 2012).

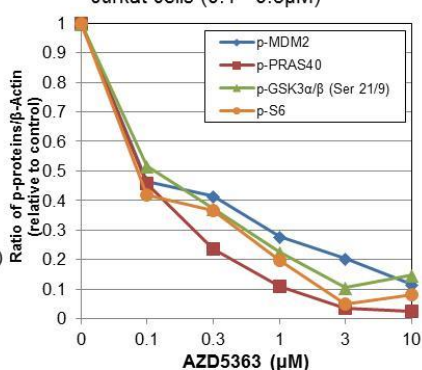
A. AZD5363 in Jurkat cells



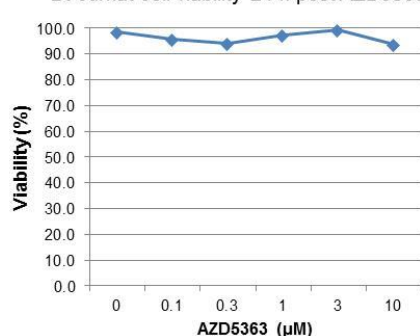
B. AZD5363 in Jurkat cells



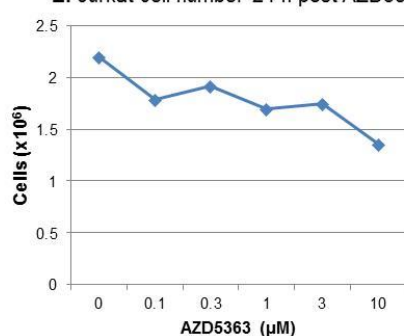
C. Estimated IC₅₀ of AZD5363 in Jurkat cells (0.1 - 0.3 μM)



D. Jurkat cell viability 24 h post AZD5363



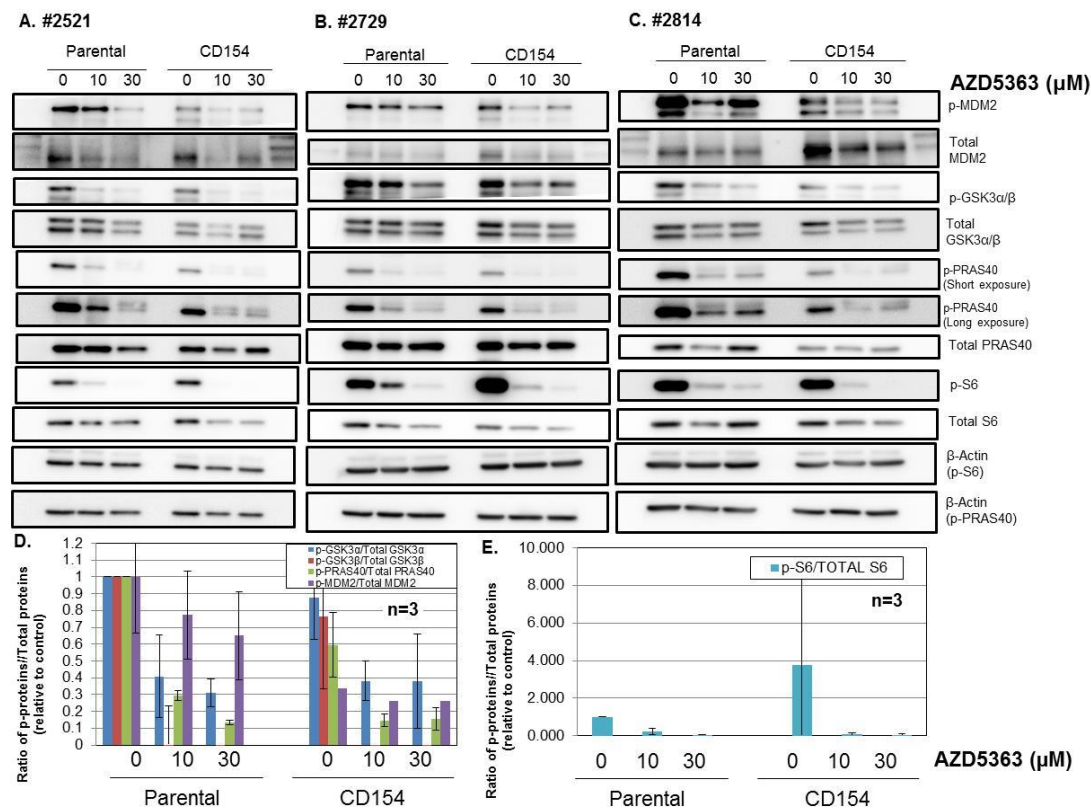
E. Jurkat cell number 24 h post AZD5363



Appendix Figure 2: Characterisation of the biological activity of AZD5363 in Jurkat cells.

- Jurkat cells were incubated for either 2h or 24h at 1×10^6 cells/ml with AZD5363 at the indicated concentrations and then examined by Western blotting for levels of phosphorylated GSK3α/β, MDM2 and total levels of GSK3β and MDM2. β-actin was used as a loading control.
- Jurkat cells were incubated for 24h at 1×10^6 cells/ml with AZD5363 at the indicated concentrations and then examined by Western blotting for levels of phosphorylated GSK3α/β (Ser 9/21), MDM2 (Ser 166), PRAS40 (Thr246) and S6 (Ser 235/236). β-actin was used as a loading control.
- Densitometry analysis of the signals corresponding to phosphorylated proteins relative to β-actin shown in B was used to quantify the effect of AZD5363 on AKT activity in Jurkat cells.
- Jurkat cell number was recorded after incubation for 24h at 1×10^6 cells/ml with AZD5363 at the indicated concentrations using the Cellometer (Cellometer Auto T4 Cell Viability Counter, Nexcelom).
- Jurkat cell viability was recorded after incubation for 24h at 1×10^6 cells/ml with AZD5363 at the indicated concentrations using 0.1% Trypan Blue /PBS [Trypan Blue solution, 0.4%, liquid, (T8154,SIGMA)] using the Cellometer (Cellometer Auto T4 Cell Viability Counter, Nexcelom).

2.2 AZD5363 is still active in CLL cells co-cultured with CD154-expressing cells.



Appendix Figure 3: Effect of AZD5363 on AKT activity in CD40-stimulated CLL cells

CLL cells were co-cultured for 24 h with control or CD154-expressing (CD154+) fibroblasts in the presence or absence of AZD5363 at the indicated concentrations and then examined by Western blotting. **A-C.** Western blotting for levels of phosphorylated and total MDM2, GSK3α/β, PRAS40 and S6 as a measure of AKT activity. β-Actin was used as a loading control. **D.** Densitometric analysis of the signals corresponding to figure 7A-C. **E.** Densitometric analysis of the signals corresponding to phospho- and total S6 in figure 7A-C.

3. Chapter 4 Supplementary: The role of AKT in antigen-independent proliferation of CLL cells

3.1 Measuring DNA synthesis after CD154 + IL-21 stimulation

We saw induction of cyclin A2 by day 3, indicating that cells were beginning to enter S-to-G2 phase ([section 4.10](#)). We also saw that AKT inhibitors inhibit the CLL cell growth induced by CD154 + IL-21 ([section 4.7](#)). Given these observations, we asked, do AKT inhibitors inhibit DNA synthesis? Do AKT inhibitors inhibit the S phase of cell cycle? To answer this question, I decided to use a non-radioactive, DNA incorporation method of BrdU. BrdU is incorporated into the DNA of proliferating cells. The cells are then fixed, and the BrdU detected by an anti-BrdU antibody conjugated to peroxidase.

Each sample was performed in triplicate. Stimulation of CLL cells with CD154 +IL-21 resulted in varied absorbance readings in different cases, therefore the data was normalised back to the CLL cells with CD154 +IL-21, to allow for comparison between cases.

The three cases used in this study were #3308 (a re-bleed of #3106), #3365 and #3355. We previously showed #3365's proliferation as measured by CFSE was inhibited by 10 μ M AZD5363 (Figure 4.13C, Figure 4.14A) and case #3308 is a re-bleed from the same patient as #3106, which was also inhibited by 10 μ M AZD5363 (Figure 4.13B, Figure 4.14A). We previously showed that #3355's proliferation as measured by CFSE was not inhibited by 10 μ M AZD5363 (Figure 4.13D, Figure 4.14C) ([section 4.6](#)).

We hoped that we might observe some difference in the absorbance readings and proliferation rate of the #3365 whose proliferation was inhibited by AZD5363, and #3355 whose proliferation wasn't.

In all cases, there appeared to be little difference in the absorbance readings, with or without either drug (AZD5363 or MK-2206). Only the addition of MK-2206 (10 μ M) saw a decrease [0.8 ± 0.37] in absorbance readings relative to the control, and this was largely due to #3308 (Appendix Figure 4A&B).

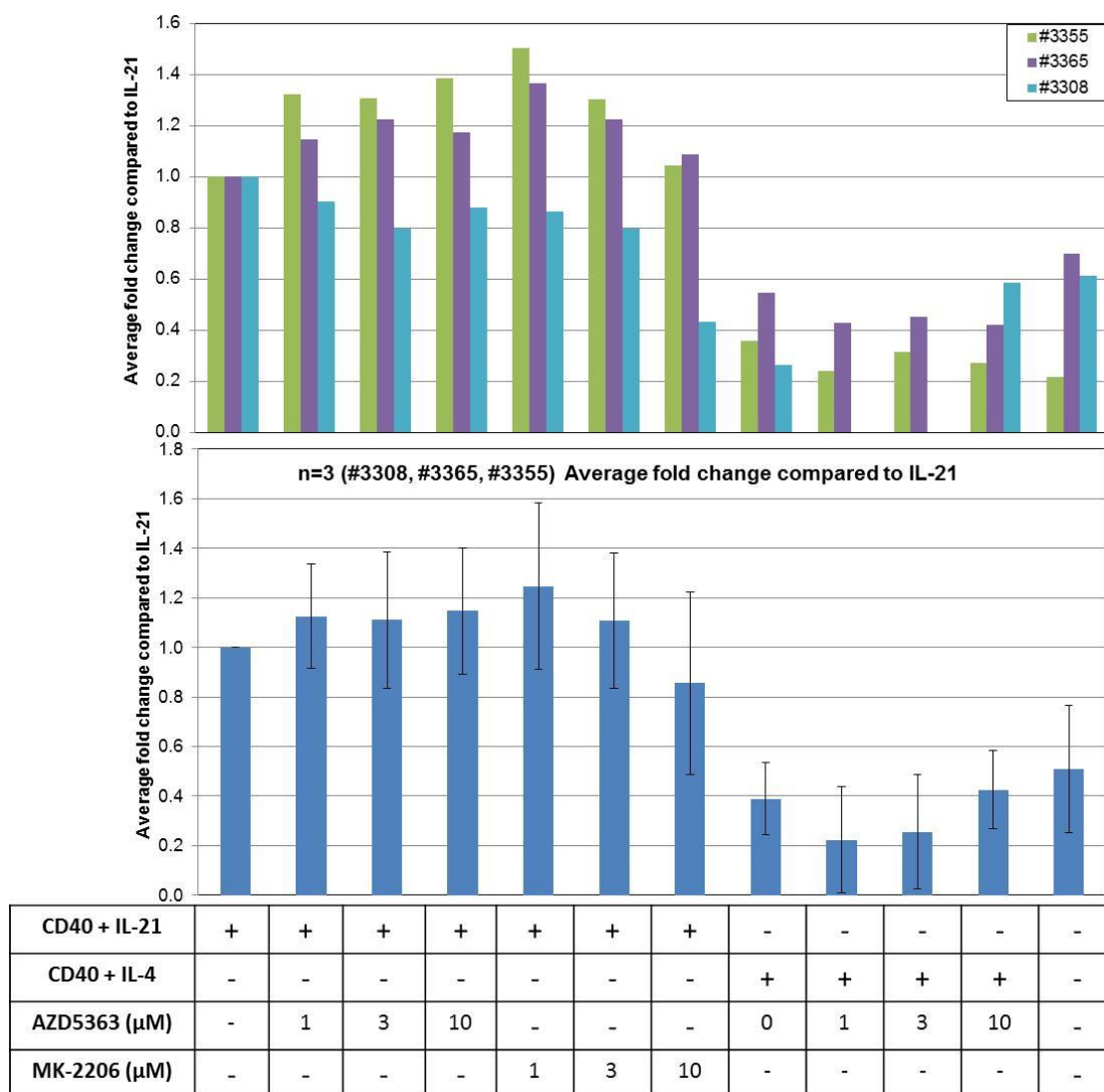
Cells plated on CD154 with the addition on IL-4, had substantially lower absorbance readings, 0.39 ± 0.14 compared to cells plated on CD154 + IL-21(Appendix Figure

4). This might indicate, either CD154 + IL-4 stimulated CLL cells synthesise DNA a lot slower than CD154 + IL-21 stimulated CLL cells, or suggest that they had not yet begun to synthesise DNA.

Background readings with no cells appeared to be quite high 0.51 ± 0.26 , which is even slightly higher than CLL cells with IL-4 (Appendix Figure 4). In light of this, we performed additional controls, with and without anti-peroxidase antibody, and BrdU as well as with and without cells (Appendix Figure 5). When no Anti-POD antibody was included, there was no absorbance. In cases with or without BrdU, the relative absorbance relative to the IL-21 control was similar to that of CLL cells supplemented with IL-4. This implied that CLL cells on the CD154 monolayer +IL-4 were not synthesising DNA. To try & reduce the background, we tried to block for 30 minutes, prior to adding the anti-peroxidase antibody, however, the results were largely the same (Appendix Figure 5).

It is worth noting, that when lenalidomide (10 μ M) was included in this assay as a positive control for the inhibition of proliferation in this BrdU system, DNA synthesis did appear to be inhibited compared to the IL-21 control to 0.476 with block, and 0.732 without block (Appendix Figure 5). So it appears as if the BrdU assay did work.

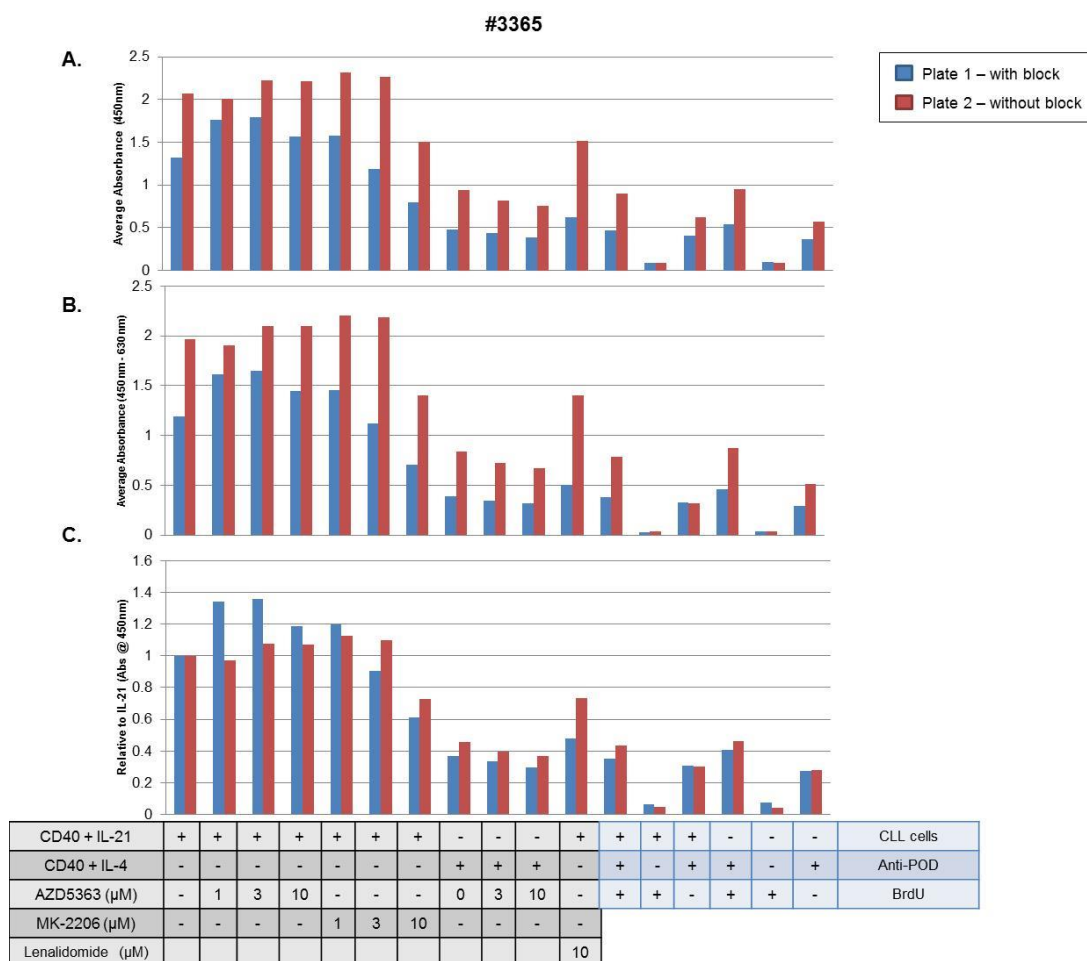
Whilst these results suggest that the AKT inhibitors do not inhibit DNA synthesis, as measured on day 3, since, the background absorbance readings in this co-culture system were still high, to be fully confident of the results, we would need to remove CLL cells from the monolayer, and examine DNA synthesis in CLL cells alone, without the presence of the monolayer to distort results. This might be best performed using a different DNA incorporation method, such as EdU which can be examined by flow cytometry.



Appendix Figure 4: BrdU incorporation data

CLL cells were supplemented with 15 ng/ml recombinant Human IL-21 (Cat. no. PHC0215, Gibco®, Life Technologies Paisley, UK) prior to plating out onto gamma irradiated CD154 fibroblasts in 96- well plates as described in materials and methods. BrdU labelling solution was added on day 1. On day 3, cells were fixed and denatured, incubated with Anti-BrdU POD, washed three times with PBS and the substrate reaction was allowed to proceed for between 5-30 minutes, then 1M H2SO4 Stop Solution was used to neutralise the reaction. The plates were read at 450nm. All, as per manufacturers protocol (Cell Proliferation ELISA, BrdU (colorimetric) (Cat. no. 11647229001, Roche).

- A. Individual cases.
- B. n=3 ± standard deviation



Appendix Figure 5: BrdU data with and without block

CLL case #3365 was incubated with indicated cytokines and drugs, on day 1, prior to plating out onto gamma irradiated CD154 fibroblasts in 96- well plates. BrdU labelling solution was added on day 1. On day 3, cells were fixed and denatured. Plate 1, was incubated with blocking buffer (Blocking Reagent For ELISA, 11112589001, made up in PBS, Roche Diagnostics Ltd) for 30 min (**blue bars on histogram**), plate 2 was not incubated with block (**red bars on histogram**). Incubated with Anti-BrdU POD, washed three times with PBS and the substrate reaction was allowed to proceed for between 5-30 minutes, then 1M H₂SO₄ Stop Solution was used to neutralise the reaction. The plates were read at 450nm (& 630nm as a control).

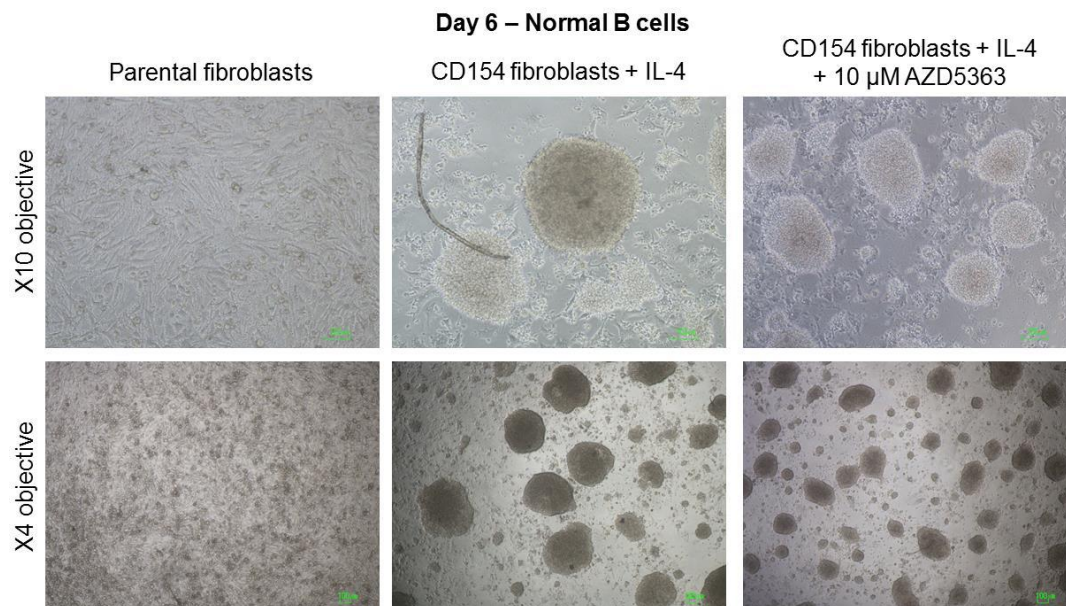
- Average absorbance (n=3) readings at 450nm
- Average absorbance (n=3) readings at 450nm minus 630nm absorbance readings.
- 450nm absorbance data normalised relative to CLL cells stimulated with IL-21 upon the CD154 monolayer

3.2 AKT inhibition affects cell-cell clustering in co-culture proliferation assays

In many of the proliferation assays which were performed, it was noted that over time, both normal B and CLL cells on CD154 + IL-4 or IL-21 began to cluster together. In broad terms, the addition of AKT inhibitors were observed to inhibit this cell clustering. This clustering effect is probably cell-cell contact mediated via integrins. Integrins, activate integrin-linked kinase (ILK) which in turn activate AKT via phosphorylation on serine 473 (McDonald et al., 2008, Persad et al., 2001), thus AKT inhibitors might reduce cell-cell contact and hence reduce cell clustering.

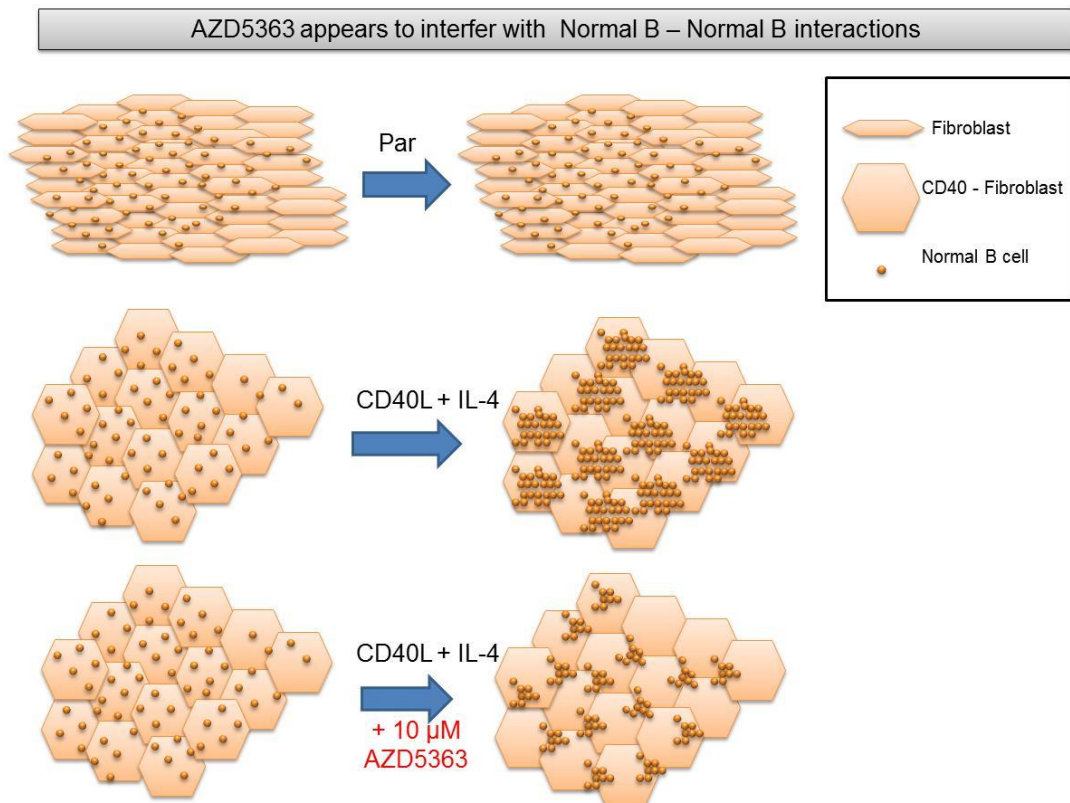
3.2.1 AKT inhibition affects Normal B cell- Normal B cell clustering in co-culture proliferation assays

In the normal B cell CD154 + IL-4 proliferation assays which were performed, it was observed that over time that normal B cells on CD154 + IL-4 began to cluster together to form clumps (Appendix Figure 6). AZD5363 (10 μ M) was observed to inhibit this cell clustering, causing smaller clumps (Appendix Figure 6 and Appendix Figure 7).



Appendix Figure 6: AZD5363 appears to interfere with Normal B – Normal B interactions.

Photos of normal B cells co-cultured with either parental fibroblasts, or CD154 fibroblasts + IL-4, or CD154 fibroblasts + IL-4 + 10 μ M AZD5363. Cells were photographed in centrally located fields of view, at high magnification (x4 objective) and at low magnification (x10 objective) using a Nikon Eclipse TS 100 microscope attached to a Nikon DS-Fi1-L2 digital camera (Nikon, Kingston upon Thames, UK). Scale bar is 100 μ M.



Appendix Figure 7: AZD5363 appears to interfere with Normal B – Normal B interactions diagram

Diagram showing that appearance of normal B cells co-cultured with either parental fibroblasts, or CD154 fibroblasts + IL-4, or CD154 fibroblasts + IL-4 + 10 μ M AZD5363. CD154 fibroblasts + IL-4 stimulation causes CLL cells to cluster. The addition of 10 μ M AZD5363, reduces the size of the clusters.

3.2.2 AKT inhibition affects CLL cell- CLL cell clustering in co-culture proliferation assays

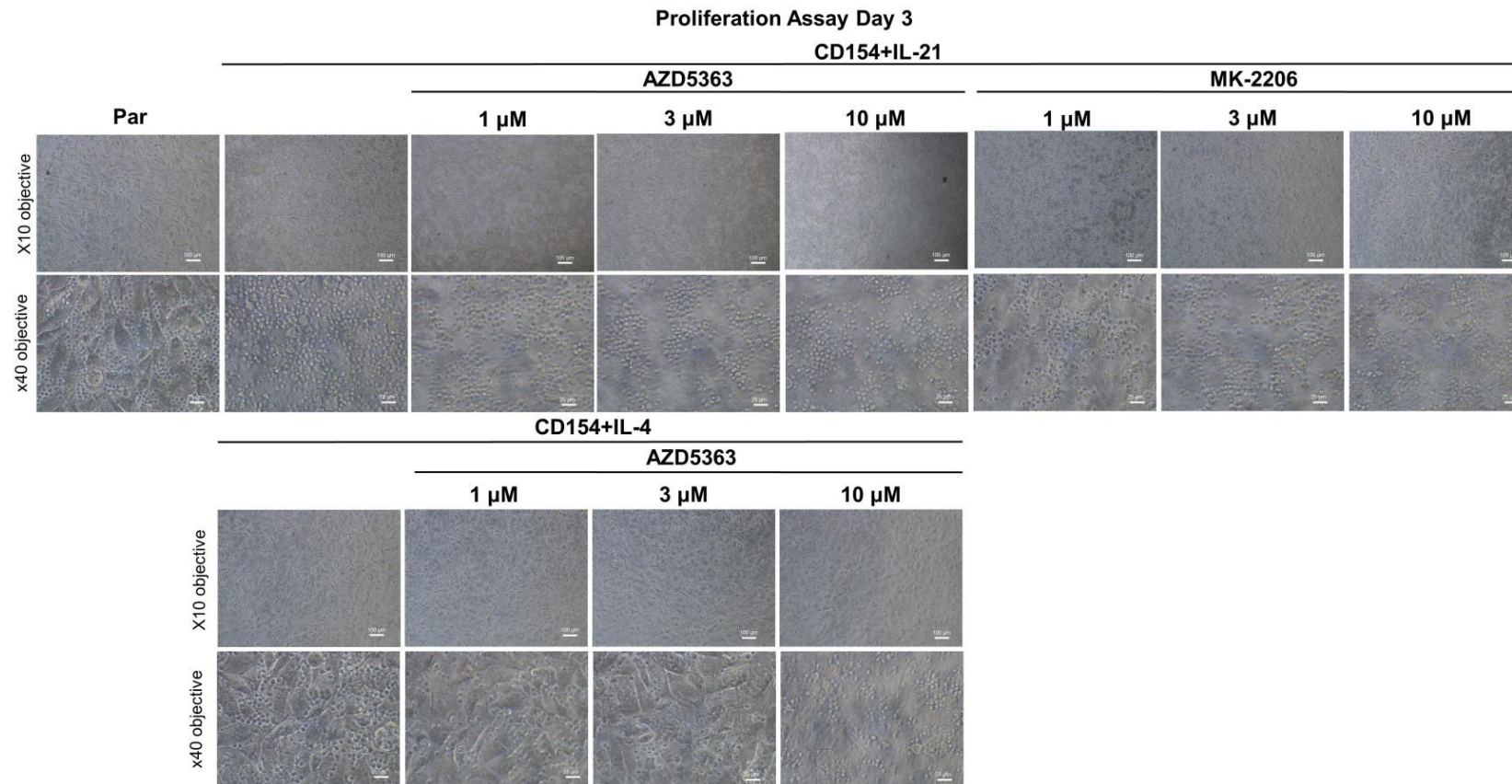
The effect of AKT inhibition described in reducing normal B cell- normal B cell interaction was also observed in the clustering of CLL-CLL cells in co-culture proliferation assays.

In order to observe this phenomena, one case of CLL, #3365 was incubated for up to seven days on either parental (Par) fibroblasts or CD154-fibroblasts (CD154) with 10 ng/ml IL-4 or 15 ng/ml IL-21 plus the indicated concentrations of AKT inhibitors. On day 3 the media was replaced with fresh media containing cytokines and drugs. Finally cells were fixed using 4% PFA, before taking photos using a microscope attached to a digital camera.

Examining CLL cells stimulated with CD154 + IL-21 on day 3 (x40 objective), you see clustering of CLL cells and addition of AZD5363 at 1 μ M, 3 μ M, 10 μ M, you see decreasing clustering. The addition of MK-2206 at 1 μ M, 3 μ M also appears to decrease clustering (Appendix Figure 8, top panel).

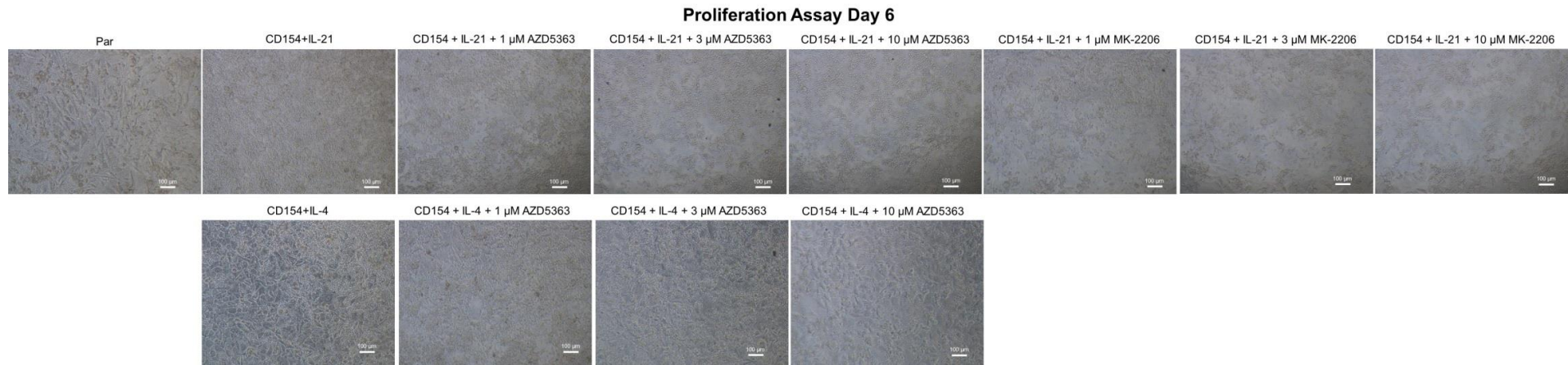
Examining CLL cells stimulated with CD154 + IL-4 on day 3, there are far fewer CLL cells in CD154 + IL-4 than CD154 + IL-21, the reason for this is unknown. It could be speculated that either CD154 + IL-21 preserves more CLL cells viable than CD154 + IL-4, or that CD154 + IL-4 stimulated CLL cells migrate under the fibroblasts, more than CLL cells stimulated with CD154 + IL-21. With the presented data, it is difficult to prove either if either of these speculations are correct. Due to the small number of CLL cells visible in CD154 + IL-4, it is difficult to see the clustering effect, let alone the effect on clustering on the addition of AZD5363 (Appendix Figure 8 bottom panel).

Examining CLL cells stimulated with CD154 + IL-21 or IL-4 on day 6, (at the lower magnification of the x10 objective), you see clustering of CLL cells, which the addition of AZD5363 or MK-2206 reduces (Appendix Figure 9).



Appendix Figure 8: Proliferation Assay Day 3 - #3365

#3365 CLL cells were incubated for three days on either parental (Par) fibroblasts or CD154-fibroblasts (CD154) with 10 ng/ml IL-4 or 15 ng/ml IL-21 plus the indicated concentrations of AKT inhibitors. Media was removed, cells were washed once with PBS, prior to fixing with 4% PFA for 30 minutes at room temperature, cells were then washed once more with PBS. Cells were photographed in centrally located fields of view, at high magnification (x40 objective) and at low magnification (x10 objective) using a Nikon Ecipse TS 100 microscope attached to a Nikon DS-Fi1-L2 digital camera.



Appendix Figure 9: Proliferation Assay Day 6 - #3365

#3365 CLL cells were incubated for six days on either parental (Par) fibroblasts or CD154-fibroblasts (CD154) with 10 ng/ml IL-4 or 15 ng/ml IL-21 plus the indicated concentrations of AKT inhibitors. On day 3 the media was replaced with fresh media containing cytokines and drugs. Media was removed, cells were washed once with PBS, prior to fixing with 4% PFA for 30 minutes at room temperature, and cells were then washed once more with PBS. Cells were photographed in centrally located fields of view, at high magnification (x40 objective) and at low magnification (x10 objective) using a Nikon Eclipse TS 100 microscope attached to a Nikon DS-Fi1-L2 digital camera.

3.3 The effect of inhibiting AKT on cell metabolism

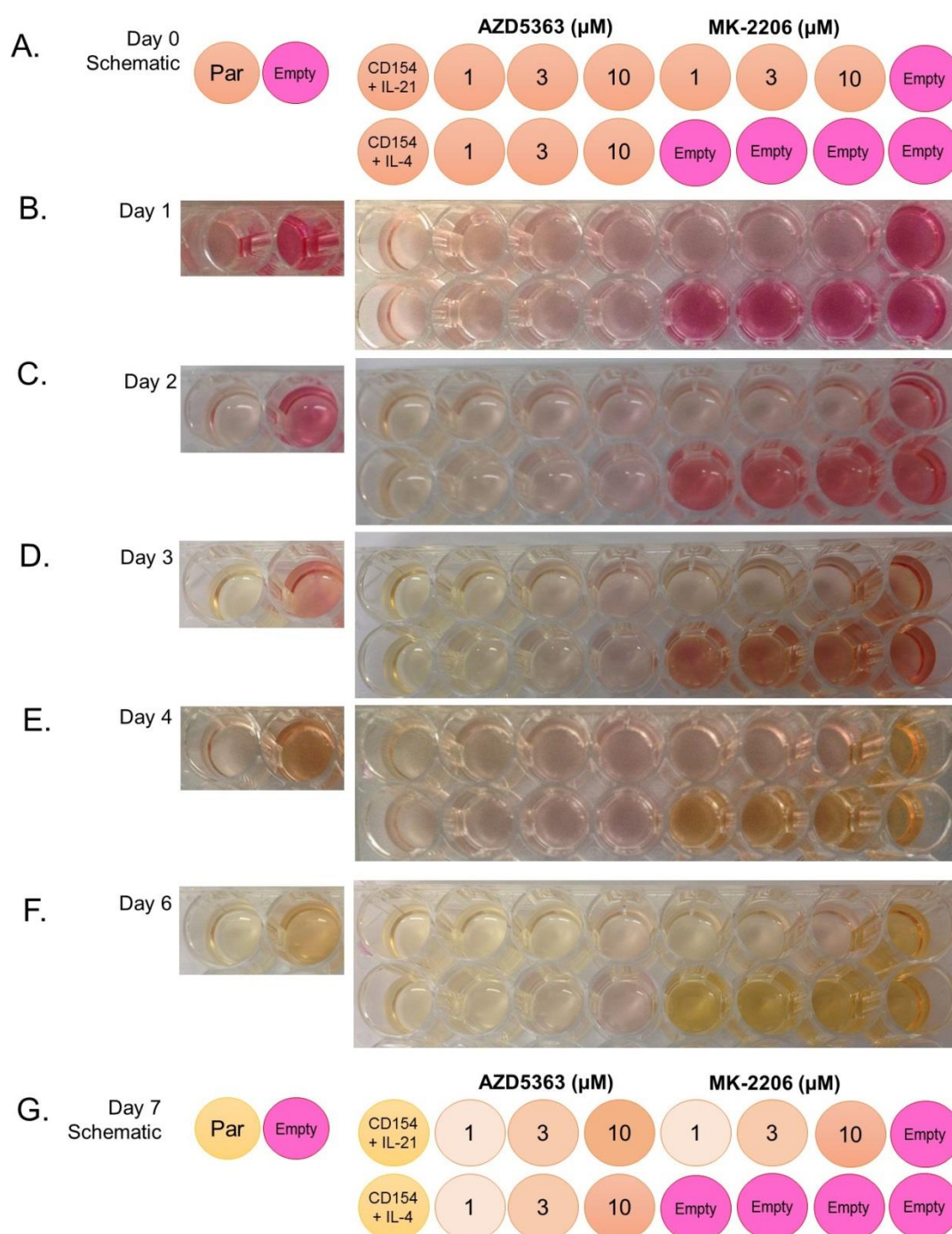
CFSE-stained primary CLL cells were co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-21 (15 ng/ml) (CD154 + IL-21) or IL-4 (10 ng/ml) (CD154 + IL-4) in RPMI media which contains phenol-red, a pH indicator. Phenol red exhibits a gradual colour transition from pink to yellow over the pH range 8.2 to 6.8 (Appendix Figure 11).

When using either proliferation assays (CD154 + IL-21 or CD154 + IL-4), it was noticeable that the RPMI media changed colour over time (days 1-7). As CLL cells grow and divide, they produce waste products, slowly the pH of the media decreases, gradually turning the solution from pink to yellow colour (Appendix Figure 10).

The addition of AKT inhibitors, AZD5363 or MK-2206 to proliferation assays (both in CD154 + IL-21 and CD154 + IL-4 based assays), inhibited the change in RPMI media colour in a dose dependent fashion when compared to the control CLL cells stimulated to divide without drug (Appendix Figure 10). The photos from days 1 – 6 to show this (Appendix Figure 10B-F). Media was changed on day 3.

Appendix Figure 10A is a schematic showing lay-out of CLL cells in 48-well format before incubation. Appendix Figure 10G is a schematic showing lay-out of CLL cells in 48-well format, showing approximately the colour change that occurred. Sadly, this colour change was not quantifiable.

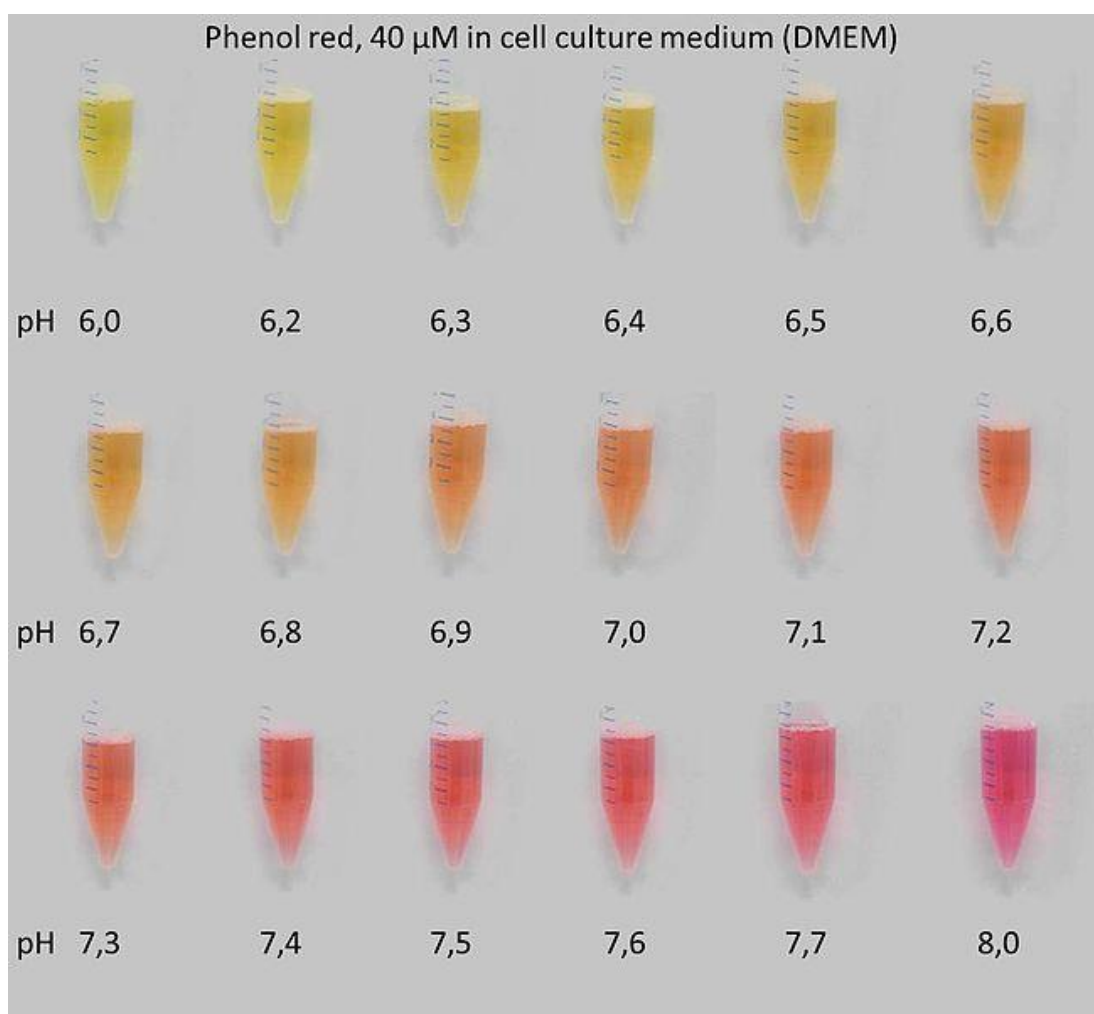
Since the colour of the media, is an indicator of pH, it is essentially measuring the amount of waste products, which is an indirect measure of cell growth and metabolism. One might therefore conclude that AKT inhibitors inhibit cellular metabolism in CLL, which would fit with the theory (see intro section '1.3.4.4 Glucose metabolism'). To fully understand how & where AKT inhibitors act to inhibit metabolism, further studies would be required.



Appendix Figure 10: The effect of AZD5363 upon media colour in CLL cell proliferation assays (CD154 + IL-21 and CD154 + IL-4).

CFSE-stained primary CLL cells were co-cultured with CD154-expressing fibroblasts in the presence of human recombinant IL-21 (15 ng/ml) (CD154 + IL-21) or IL-4 (10 ng/ml) (CD154 + IL-4) and AKT inhibitors, AZD5363 or MK-2206 at the indicated concentrations. Media was changed on day 3. CLL cells co-cultured with parental fibroblasts (Par) were used as a negative control.

- A. Day 0 - Schematic showing lay-out of CLL cells in 48-well format.
- B. Day 1 - Photo of cells.
- C. Day 2 - Photo of cells.
- D. Day 3 - Photo of cells.
- E. Day 4 - Photo of cells.
- F. Day 6 - Photo of cells.
- G. Day 7 - Schematic showing lay-out of CLL cells in 48-well format, showing approximately the colour change to media that occurs



Appendix Figure 11: Phenol red, 40 μ M: colours in cell culture medium at a pH range from 6.0 to 8.0.

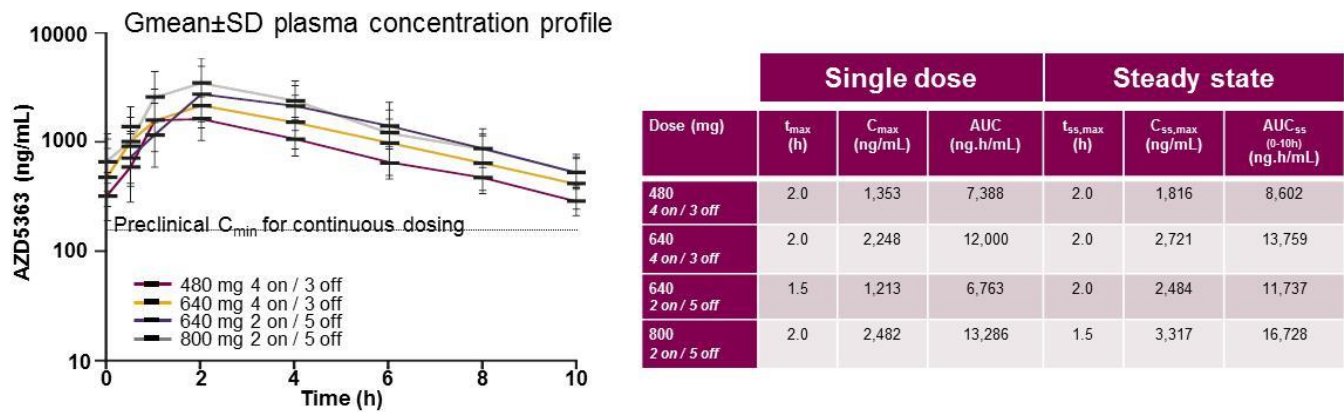
(Source: http://en.wikipedia.org/wiki/Phenol_red)

4. Data provided kindly by AstraZeneca

Appendix Figure 12: AZD5363 PK profile in Western patients receiving intermittent bid dosing (Banerji, 2013).

480 mg BID (steady state), gives $C_{max} = 1816 \text{ ng/mL} = 4.23 \text{ }\mu\text{M}$.
800 mg BID (steady state), gives $C_{max} = 3317 \text{ ng/mL} = 7.7 \text{ }\mu\text{M}$.
480 mg bid is the recommended phase 2 dose which is being used in most the clinical studies with this drug.

AZD5363 PK profile in Western patients receiving intermittent bid dosing



PK following a single dose

- AZD5363 is rapidly absorbed with half-life of approx 10 hours
- Exceeds exposure required for preclinical efficacy based on preclinical modeling
- Dose proportional increase in C_{max} /AUC

AUC_{ss}: steady state area under the curve; $C_{ss,max}$: time to C_{max} at steady state; t_{max} : time to C_{max} ; $t_{ss,max}$: time to maximum plasma concentration at steady state



Appendix Table 2: Potency of PI3K inhibitors – AstraZeneca data

Data kindly provided by AstraZeneca, unpublished data produced by AstraZeneca in house.

Compound	Enzyme IC ₅₀ (nM)				Cell IC ₅₀ (uM)			
	α	β	δ	γ	α driven (BT474c)	β driven (MDA- MB-468)	δ driven (JEKO)	γ driven (RAW- 264)
GDC-0941	5	28	4	68	0.04	0.17	0.01	0.15
IPI-145	182	21	<1	6	0.78	0.10	<0.001	0.11
Idelalisib	715	260	4	72	12.1	0.64	0.004	1.0
	Ratios (enzyme)							
GDC-0941	1.25	7	1	17				
IPI-145	>182	>21	1	>6				
Idelalisib	179	65	1	18				

Appendix Table 3: Primary CLL cells used in each figure.

	Chapter 3														Chapter 4)														Chapter 5											Appendix			
Sample #	1	2	2	3	3	3	4	5	6	7	8	9	10		1, 2, 3	4	5	9, 10	11	12	13, 14, 15	16	17	18	22, 23	24, 25, 26		1	2	3	4, 5, 6 7	8	9	10		3	4	5	6				
1958		✓		✓	✓																								6														
2064																												7															
2096	✓			✓																									4														
2103	✓			✓								✓	✓																4														
2263		✓	✓	✓																									9														
2441																												2															
2474																												10															
2521		✓		✓	✓		✓		✓	✓	✓																		8							✓							
2674																												✓	3														
2729		✓		✓	✓		✓		✓	✓	✓				x2																					✓							
2814	✓			✓	✓	✓	✓		✓	✓	x2				x2		✓	✓				✓						✓	1							✓	✓						
3033		✓	✓	✓	✓																																		✓				
3058		✓		✓	✓						x2				✓	✓																											
3074																												✓	5														
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